GENE REGULATING AUREOBASIDIN SENSITIVITY

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1. Field of the Invention.

This invention relates to a protein regulating a sensitivity to an antimycotic aureobasidin, a gene encoding this protein and to uses of the protein and gene.

2. Description of Related Art.

Systemic mycoses including candidiasis have increased with an increase in immunocompromised patients in recent years due to, for example, the extended use of immunosuppressive drugs and acquired immunodeficiency syndrome (AIDS), and as opportunistic infection due to microbial substitution caused by the frequent use of widespectrum antibacterial antibiotics. Although drugs for treating mycoses such as amphotericin B, flucytosine and azole drugs (for example, fluconazole and miconazole) are now used to cope with this situation, none of them can achieve a satisfactory effect. Also, known diagnostic drugs are insufficient. For candidiasis, in particular, although there have been known several diagnostic drugs (for example, CAND-TEC for detection of candida antigen and LABOFIT for detection of D-arabinitol), none of them gives any satisfactory results in specificity or sensitivity.

The reasons for the delay in the development of remedies and diagnostic drugs for mycoses as described above are that fungi causing the mycoses are eukaryotic organisms similar to the host (i.e., man) and thus are not largely different from man and that knowledges of fungi, in particular, pathogenic fungi are insufficient. Therefore it is difficult to distinguish fungi from man or to selectively kill fungi, which is responsible for the delay in the development of drugs for mycoses.

Recently, the application of genetic engineering techniques such as antisense or PCR to the treatment and diagnosis of mycoses has been expected. However known genes which are applicable thereto and/or proteins coded for by these genes are rare (PCT Pamphlet W092/03455). Regarding

pathogenic fungi, there have been cloned in recent years an acid protease gene, which has been assumed to participate in the pathogenicity of *Candida albicans* (hereinafter referred to simply as *C. albicans*) and *Candida tropicalis* (hereinafter referred to as *C. tropicalis*) causing candidiasis [B. Hube et al., J. Med. Vet. Mycol., 29, 129 - 132 (1991); Japanese Patent Laid-Open No. 49476/1993; and G. Togni et al., FEBS Letters, 286, 181 - 185 (1991)], a calmodulin gene of *C. albicans* [S.M. Saporito et al., Gene, 106, 43 - 49 (1991)] and a glycolytic pathway enzyme enolase gene of *C. albicans* [P. Sundstrom et al., J. Bacteriology, 174, 6789 - 6799 (1991)]. However, each of these genes and proteins coded for thereby is either indistinguishable from nonpathogenic fungi and eukaryotic organisms other than fungi or, if distinguishable therefrom, cannot serve as a definite action point for exhibiting any selective toxicity.

Aureobasidin [Japanese Patent Laid-Open No. 138296/1990, No. 22995/1991, No. 220199/1991, No. 279384/1993, and No. 65291/1994; J. Antibiotics, 44 (9), 919 - 924, ibid., 44 (9), 925 - 933, ibid., 44 (11), 1187 - 1198 (1991)] is a cyclic depsipeptide obtained as a fermentation product of a strain *Aureobasidium pullulans* No. R106. It is completely different in structure from other antimycotics. As Tables 1 and 2 show below, aureobasidin A, which is a typical aureobasidin compound, exerts a potent antimycotic activity on various yeasts of the genus *Candida* including *C. albicans* which is a pathogenic fungus, *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Blastomyces dermatitidis* and fungi of the genus *Aspergillus* and *Penicillium* (Japanese Patent Laid-Open No. 138296/1990) but has an extremely low toxicity in mammal. Thus this compound is expected to be useful as an antimycotic being excellent in selective toxicity.

Hereinafter, *Candida*, *Cryptococcus* and *Aspergillus* will be abbreviated respectively as *C.*, *Cr.* and *A.*

Table 1

Test Strain	TIMM No.	MIC(μg/ml)
C. albicans	0136	≤ 0.04
C. albicans var. stellatoidea	1308	≤ 0.04
C. tropicalis	0312	0.08
C. kefyr	0298	0.16
C. parapsilosis	0287	0.16
C. krusei	0270	≤ 0.04
C. guilliermondii	0257	0.08
C. glabrata	1062	≤ 0.04
Cr. neoformans	0354	0.63
Cr. terreus	0424	0.31
Rhodotorula rubra	0923	0.63
A. fumigatus	0063	20
A. clavatus	0056	0.16

Table 2

Test Strain	TIMM No.	MIC(μg/ml
A. nidulans	0112	0.16
A. terreus	0120	5
Penicillium commune	1331	1.25
Trichophyton mentagrophytes	1189	10
Epidermophyton floccosum	0431	2.5
Fonsecaea pedrosoi	0482	0.31
Exophiala werneckii	1334	1.25
Cladosporium bantianum	0343	0.63
Histoplasma capsulatum	0713	0.16
Paracoccidioides brasiliensis	0880	0.31
Geotrichum candidum	0694	0.63
Blastomyces dermatitidis	0126	0.31

Each of the existing antimycotics with a low toxicity shows only a fungistatic action, which causes a clinical problem. In contrast, aureobasidin exerts a germicidal action. Although it has been required to clarify the mechanism of the selective toxicity of aureobasidin from these viewpoints, this mechanism still remains completely unknown.

As described in Canadian Patent Laid-Open No. 2124034, the present inventors have previously found out that *Saccharomyces cerevisiae* (hereinafter referred to simply as *S. cerevisiae*) and *Schizosaccharomyces pombe* (hereinafter referred to simply as *Schizo. pombe*) are sensitive to aureobasidin. We have further mutated sensitive cells of *S. cerevisiae* or *Schizo. pombe* into resistant cells and successfully isolated a gene capable of imparting a resistance to aureobasidin (a resistant gene) therefrom. We have furthermore successfully isolated a gene capable of imparting aureobasidin sensitivity (a sensitive gene) from the corresponding sensitive cells.

We have also isolated a gene regulating aureobasidin sensitivity from *C. albicans* with the

use of the gene regulating aureobasidin sensitivity or a part thereof as a probe. However no gene regulating aureobasidin sensitivity has been found in molds including those belonging to the genus *Aspergillus*.

There have been known techniques for introducing useful genes into monoploid fungal cells to be used in a laboratory, for example, *Saccharomyces cerevisiae* (hereinafter referred to simply as *S. cerevisiae*), *Schizosaccharomyces pombe* (hereinafter referred to simply as *Schizo. pombe*) and *Aspergillus nidulans* (hereinafter referred to simply as *A. nidulans*). Since the incorporation and fixation of plasmid DNAs into fungal cells are relatively scarcely successful, it is required to use selective markers in the identification of transformants. In the most common case, selection can be achieved by introducing an auxotrophic mutation into host cells. Examples of the mutation generally employed in, for example, *S. cerevisiae* include ura3, leu2, trp1 and his3. A plasmid carries a wild type copy of one of these genes. Since the wild type copy on the plasmid is dominant over the chromosomal allele of the host, cells having the plasmid introduced thereinto can be screened in a minimal medium which contains no nutrient required by the auxotrophic host cells. Also there have been published some reports, though in a small number, relating to the use of drug resistance in the screening of transformants. Namely, there have been reported replication vectors and chromosome integration vectors containing genes which are resistant against antibiotics such as a neomycin homologue G418, hygromycin and cerulenin. A replication vector has a DNA replication origin acting in a cell. This plasmid is held outside the chromosome as a cyclic episome and continuously reduced at a ratio of several percent with the proliferation of the cells. An integration vector is inserted into the chromosome of a host cell and thus held in a stable state. In this case, therefore, it is unnecessary to further add a drug to the medium in order to exert the selection function for maintaining the sequence of the vector.

In the case of industrial fungi, it is required to sustain the useful character, which has been imparted thereto, in a stable state. A chromosome integration vector is useful for this purpose.

Fungi have been widely applied to the production of liquors such as sake, beer and wine and fermented foods such as miso (fermented soy bean paste) and soy sauce. For breeding these fungi to be used for industrial purposes, genetic engineering techniques are also highly effective in order to impart useful characteristics thereto. Thus there have been required selective markers which are

usable in efficiently screening transformants. Industrial yeasts are usually di- or polyploid cells. It is therefore difficult to introduce an auxotrophic marker, which is effective in monoploid cells of, for example, yeasts to be used in a laboratory, into these industrial yeasts. In addition, since there is a high possibility that a mutagenesis induces mutation in other genes, accordingly, it is highly difficult to create a mutant having the desired auxotrophic mutation alone introduced thereinto. The use of a drug resistance makes it possible to screen a stable transformant of an arbitrary yeast regardless of the number of chromosomes or the occurrence of specific mutation. However many of these industrial fungi are insensitive to antibiotics such as G-418 and hygromycin, which makes it impossible to use genes resistant against these antibiotics therefor. Moreover, these resistant genes are genes or proteins derived from bacteria which are procaryotes, and none of them corresponding to these genes is present in fungi such as yeasts. The use of fungal cells having these foreign genes integrated therein is seriously restricted. A cerulenin resistant gene (PDR4) originating in *S. cerevisiae* is usable in the transformation of *S. cerevisiae* including brewing yeast. However it also conferred resistances against drugs other than cerulenin, which might bring about some problems in the practical use. Therefore PDR4 cannot fully satisfy the requirements for breeding industrial fungi including *S. cerevisiae* having improved characters in the future. Thus it has been required to develop drug resistant markers with the use of genes which are inherently carried by fungi.

There are a number of molds such as the ones of the genera *Aspergillus* and *Penicillium*. Some of these molds have been applied to food manufacturing (for example, brewing of liquors, soy sauce and miso, ripening of cheese, etc.) for a long time, while a number of them are important in the production of enzyme preparations or antibiotics. However, molds include not only these useful ones as described above but also harmful ones such as those inducing plant diseases and those causing serious human diseases such as deep-seated mycosis. The recent development in genetic engineering techniques has made it possible not only to breed useful strains but also to apply molds to novel purposes, for example, the production of a heterogenic protein. Also, analyses of vital phenomena of molds are under way.

An object of the present invention is to find a gene, which encodes a protein regulating aureobasidin sensitivity and which is useful in genetic engineering techniques and in analyses of vital

phenomena of molds from molds including those belonging to the genus *Aspergillus* and its functional derivative. That is to say, the present invention aims at revealing a gene which encodes a protein regulating aureobasidin sensitivity or its functional derivative; providing a method for cloning this gene and a protein regulating aureobasidin sensitivity encoded by this gene or its functional derivative; providing the antisense DNA and the antisense RNA of this gene; providing a nucleic acid probe hybridizable with this gene and a method for detecting this gene by using this nucleic acid probe; and providing a process for producing a protein regulating aureobasidin sensitivity or its functional derivative by using this gene.

Under these circumstances, the present invention further aims at finding a novel protein regulating aureobasidin sensitivity through the clarification of the mechanism of the selective toxicity to fungi of aureobasidin. Accordingly, the present invention aims at finding a gene coding for a protein regulating aureobasidin sensitivity, providing a process for cloning this gene and the protein regulating aureobasidin sensitivity which is encoded by this gene, further providing an antisense DNA and an antisense RNA of this gene, providing a nucleic acid probe being hybridizable with this gene, providing a process for detecting this gene with the use of the nucleic acid probe, providing a process for producing the protein regulating aureobasidin sensitivity by using this gene and providing an antibody against the protein regulating aureobasidin sensitivity, and a process for detecting the protein regulating aureobasidin sensitivity by using this antibody.

In addition, the present invention aims at providing a novel chromosome integration vector capable of imparting a novel selective marker of a drug resistance to a fungal transformant, and a transformant transformed by this vector.

The present invention further aims at providing a protein capable of imparting the aureobasidin resistance and acting as a selective marker which is usable in genetic engineering of fungi, and a DNA coding for this protein.

SUMMARY OF THE INVENTION

The present invention may be summarized as follows. Namely, the first invention of the present invention relates to an isolated gene coding for a protein regulating aureobasidin sensitivity, that is, a gene regulating aureobasidin sensitivity. The second invention relates to a process for cloning a gene regulating aureobasidin sensitivity which is characterized by using the gene regulating

aureobasidin sensitivity of the first invention or a part thereof as a probe. The third invention relates to a nucleic acid probe which is hybridizable with a gene regulating aureobasidin sensitivity and comprises a sequence consisting of 15 or more bases. The fourth invention relates to an antisense DNA of a gene regulating aureobasidin sensitivity. The fifth invention relates to an antisense RNA of a gene regulating aureobasidin sensitivity. The sixth invention relates to a recombinant plasmid having a gene regulating aureobasidin sensitivity contained therein. The seventh invention relates to a transformant having the above-mentioned plasmid introduced thereinto. The eighth invention relates to a process for producing a protein regulating aureobasidin sensitivity by using the above-mentioned transformant. The ninth invention relates to an isolated protein regulating aureobasidin sensitivity. The tenth invention relates to an antibody against a protein regulating aureobasidin sensitivity. The eleventh invention relates to a process for detecting a protein regulating aureobasidin sensitivity by using the above-mentioned antibody. The twelfth invention relates to a process for detecting a gene regulating aureobasidin sensitivity by the hybridization which is characterized by using the nucleic acid probe of the third invention of the present invention. The thirteenth invention relates to a process for screening an antimycotic by using the above-mentioned transformant or a protein regulating aureobasidin sensitivity. The fourteenth invention of the present invention relates to a chromosome integration vector for a host fungus which is characterized by containing an aureobasidin resistant gene. This chromosome integration vector sometimes contains a foreign gene. The fifteenth invention relates to a process for producing an aureobasidin resistant transformant characterized by comprising:

(1) the step of obtaining a replication vector which contains an aureobasidin resistant gene,

(2) the step of cleaving the aureobasidin resistant gene in the replication vector obtained in the above step at one site to give a chromosome integration vector for a host fungus;

(3) the step of integrating the chromosome integration vector for a host fungus obtained in the above step into the chromosome of the host fungus; and

(4) the step of selecting a host which has been transformed into an aureobasidin resistant one in the presence of aureobasidin.

In this process for producing an aureobasidin resistant transformant, the replication vector sometimes contains a foreign gene. The sixteenth invention relates to a transformant characterized

by being one obtained by the process of the fifteenth invention.

The seventeenth invention relates to a protein capable of imparting aureobasidin resistance, wherein at least the 240th amino acid residue Ala in the protein capable of imparting aureobasidin sensitivity represented by SEQ ID No. 22 in the Sequence Listing has been replaced by another amino acid residue, or another protein capable of imparting aureobasidin resistance which has an amino acid sequence obtained by subjecting the above-mentioned protein to at least one modification selected from replacement, insertion and deletion of amino acid residue(s) and shows a biological activity comparable to that of the above-mentioned protein. The eighteenth invention relates to a DNA which codes for the protein capable of imparting the aureobasidin resistance of the seventeenth invention.

The nineteenth invention relates to a gene originating in a mold which encodes a protein regulating aureobasidin sensitivity or its functional derivative. Namely, it relates to a gene regulating aureobasidin sensitivity obtained from a mold or a functional derivative thereof. The twentieth invention relates to a method for cloning a gene regulating aureobasidin sensitivity and originating in a mold or its functional derivative wherein the gene regulating aureobasidin sensitivity of the nineteenth invention or its functional derivative is employed as a probe either as the whole or a part thereof. The twenty-first invention relates to a nucleic acid probe comprising a sequence consisting of at least 15 bases which is hybridizable with a gene regulating aureobasidin sensitivity and originating in a mold or its functional derivative. The twenty-second invention relates to the antisense DNA of a gene regulating aureobasidin sensitivity and originating in a mold or its functional derivative. The twenty-third invention relates to the antisense RNA of a gene regulating aureobasidin sensitivity and originating in a mold or its functional derivative. The twenty-fourth invention relates to a recombinant plasmid which contains a gene regulating aureobasidin sensitivity and originating in a mold or its functional derivative. The twenty-fifth invention relates to a transformant which has the plasmid of the twenty-fourth invention introduced thereinto. The twenty-sixth invention relates to a process for producing a protein regulating aureobasidin sensitivity or its functional derivative with the use of the above-mentioned transformant. The twenty-seventh invention relates to a protein regulating aureobasidin sensitivity and originating in a mold or its functional derivative. The twenty-eighth invention relates to a protein capable of imparting the

resistance to aureobasidin, wherein at least the amino acid Gly at the position 275 of the protein imparting aureobasidin sensitivity represented by SEQ ID NO. 4 in the Sequence Listing has been replaced by another amino acid, or its functional derivative. The twenty-ninth invention relates to a DNA which encodes the protein of the twenty-eighth invention capable of imparting the resistance to aureobasidin. The thirtieth invention relates to a method for detecting a gene regulating aureobasidin sensitivity by hybridization with the use of the nucleic acid probe of the twenty-first invention.

As described in Japanese Patent Application No.106158/1994, the present inventors have previously found out that fungi such as *Schizo. pombe* and *S. cerevisiae* and, further, mammalian cells such as mouse lymphoma EL-4 cells, are sensitive to aureobasidin, as Table 3 shows.

Table 3

Test Strain or Cell	MIC(μg/ml)
Schizo. pombe	0.08
S. cerevisiae	0.31
mouse lymphoma EL-4	10
mouse lymphoma L5178Y	100
NRK-49F	12.5

The present inventors have mutagenized a wild-type strain of *Schizo. pombe* or *S. cerevisiae*, sensitive to aureobasidin, to thereby give resistant mutants. We have further successfully isolated a gene capable of confering aureobasidin resistance (a resistant gene) from these resistant mutants and another gene capable of imparting aureobasidin sensitivity (a sensitive gene) from the corresponding sensitive cells. Furthermore, we have disclosed the existence of a protein encoded by each of these genes. By culturing cells which have been transformed by introducing the above-mentioned gene, we have succeeded in the expression of this gene. Furthermore, we have successfully found out a novel gene regulating aureobasidin sensitivity from another fungus being sensitive to aureobasidin by using a DNA fragment of the above-mentioned gene as a probe. In

addition, we have clarified that the gene regulating aureobasidin sensitivity is essentially required for the growth of the cells and found out that the detection of this gene or a protein which is a gene product thereof with an antibody enables the diagnosis of diseases caused by these cells, for example, mycoses induced by fungi, and that an antisense DNA or an antisense RNA, which inhibits the expression of the gene regulating aureobasidin sensitivity being characteristic to the cells, is usable as a remedy for diseases caused by these cells, for example, mycoses induced by fungi, thus completing the present invention. The present inventors have also succeeded in the expression of this gene by preparing a replication vector containing this gene and incubating cells transformed by using this vector. By using a DNA fragment of this gene as a probe, they have further successfully found a novel gene regulating the aureobasidin sensitivity from another fungus which is sensitive to aureobasidin.

The pathogenic fungi listed in Tables 1 and 2 and fungi and mammalian cells listed in Table 3, each having a sensitivity to aureobasidin; each carries a protein regulating aureobasidin sensitivity and a gene coding for this protein. The term "a protein regulating aureobasidin sensitivity" as used herein means a protein which is contained in an organism, particularly a fungus, having a sensitivity to aureobasidin. This protein is required for the expression of the sensitivity or resistance to aureobasidin. As a matter of course, a protein having 35% or more homology with the above-mentioned protein and having a similar function is also a member of the protein regulating aureobasidin sensitivity according to the present invention. Furthermore, proteins obtained by modifying these proteins by the genetic engineering procedure are members of the protein regulating aureobasidin sensitivity according to the present invention. A gene regulating aureobasidin sensitivity means a gene which codes for such a protein regulating aureobasidin sensitivity as those described above and involves both of sensitive genes and resistant genes.

The first invention of the present invention relates to a gene regulating aureobasidin sensitivity. This gene can be isolated in the following manner. First, aureobasidin sensitive cells (a wild-type strain) are mutagenized to thereby induce a resistant strain. From chromosome DNA or cDNA of this resistant strain, a DNA library is prepared and a gene capable of confering resistance (a resistant gene) is cloned from this library. Then a DNA library of a wild strain is prepared and a DNA molecule being hybridizable with the resistant gene is isolated from this library and cloned.

Thus a sensitive gene can be isolated.

The mutagenesis is performed by, for example, treating with a chemical such as ethylmethane sulfonate (EMS) or N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) or by ultraviolet or other radiation. The cell that has acquired the resistance can be screened by culturing the mutagenized cells in a nutritional medium containing aureobasidin at an appropriate concentration under appropriate conditions. The resistant strain thus obtained may vary depending on the method and conditions selected for the mutagenesis. Also, strains differing in the extent of resistance from each other can be separated by changing the aureobasidin concentration or a temperature-sensitive resistant strain can be isolated by changing the temperature in the step of screening. There are a number of mechanisms of resistance to aureobasidin. Accordingly, a number of resistant genes can be isolated by genetically classifying these various resistant strains. In the case of a yeast, the classification may be performed by the complementation test. Namely, resistant strains are prepared from haploid cells. Next, diploid cells can be obtained by crossing resistant strains differing in mating type from each other. Then spores formed from these diploids are examined by the tetrad analysis.

As typical examples of the genes regulating aureobasidin sensitivity(named aur) according to the present invention, aur1 and aur2 genes may be cited. Typical examples of the aur1 gene include spaur1 gene isolated from *Schizo. pombe* and scaur1 gene isolated from *S. cerevisiae*, while typical examples of the aur2 gene include scaur2 gene isolated from *S. cerevisiae*. Now, resistant genes (spaur1^R, scaur1^R and scaur2^R) isolated from resistant mutants by the present inventors and sensitive genes (spaur1^S, scaur1^S and scaur2^S) isolated from sensitive wild-type strains will be described.

Fig. 1 shows a restriction enzyme map of the genes spaur1^R and spaur1^S regulating aureobasidin sensitivity, Fig. 2 shows a restriction enzyme map of scaur1^R and scaur1^S and Fig. 3 shows a restriction enzyme map of scaur2^R and scaur2^S.

Schizo. pombe, which is sensitive to aureobasidin, is mutagenized with EMS and a genomic library of the resistant stain thus obtained is prepared. From this library, a DNA fragment containing a resistant gene (spaur1^R) and having the restriction enzyme map of Fig. 1 is isolated. This gene has a nucleotide sequence represented by SEQ ID No. 15 in Sequence Listing. The amino acid

sequence of a protein encoded by this gene, which is estimated on the basis of this nucleotide sequence, is the one represented by SEQ ID No. 16 in Sequence Listing. By the hybridization with the use of this resistant gene as a probe, a DNA fragment containing a sensitive gene (spaur1^S) and having the restriction enzyme map of Fig. 1 is isolated from a sensitive strain. This gene has a nucleotide sequence represented by SEQ ID No. 17 in Sequence Listing. The amino acid sequence of a protein encoded by this gene, which is estimated on the basis of this nucleotide sequence, is the one represented by SEQ ID No. 18 in Sequence Listing. A comparison between the sequences of SEQ ID No. 17 and SEQ ID No. 15 reveals that a mutation from G to T occurs at the base at the position 1053, while a comparison between the sequences of SEQ ID No. 18 and SEQ ID No. 16 reveals that glycine at the residue 240 is converted into cysteine at the amino acid level, thus giving rise to the resistance.

Also, *S. cerevisiae*, which is sensitive to aureobasidin, is mutagenized with EMS and genomic libraries of two resistant strains thus obtained are prepared. From one of these libraries, a DNA fragment containing a resistant gene (scaur1^R) as a dominant mutant and having the restriction enzyme map of Fig. 2 is isolated, while a DNA fragment containing a resistant gene (scaur2^R) and having the restriction enzyme map of Fig. 3 is isolated from another library.

The nucleotide sequence of the coding region for the protein of the scaur1^R gene is the one represented by SEQ ID No. 19 in Sequence Listing. The amino acid sequence of the protein encoded by this gene, which is estimated on the basis of the above nucleotide sequence, is the one represented by SEQ ID No. 20 in Sequence Listing. By the hybridization with the use of this resistant gene scaur1^R as a probe, a DNA fragment containing a sensitive gene (scaur1^S) and having the restriction enzyme map of Fig. 2 is isolated from a sensitive strain. This gene has a nucleotide sequence represented by SEQ ID No. 21 in Sequence Listing. The amino acid sequence of a protein encoded by this gene, which is estimated on the basis of this nucleotide sequence, is the one represented by SEQ ID No. 22 in Sequence Listing. A comparison between the sequences of SEQ ID No. 21 and SEQ ID No. 19 reveals that a mutation from T to A occurs at the base at the position 852, while a comparison between the sequences of SEQ ID No. 22 and SEQ ID No. 20 reveals that phenylalanine at the residue 158 is converted into tyrosine at the amino acid level, thus giving rise to the resistance. The spaur1 gene has a 58% homology with the scaur1 gene at the amino acid

level. Thus it is obvious that they are genes coding for proteins having similar functions to each other. When genes and proteins being homologous in sequence with the spaur1 and scaur1 genes and with the proteins encoded thereby are searched from a data base, none having a homology of 35% or above is detected. Accordingly, it is clear that these genes and the proteins encoded thereby are novel molecules which have never been known hitherto.

By the hybridization with the use of the DNA fragment of the resistant gene scaur2^R as a probe, a DNA fragment containing a sensitive gene (scaur2^S) and having the restriction enzyme map of Fig. 3 is isolated from a sensitive strain.

The nucleotide sequence of this sensitive gene is the one represented by SEQ ID No. 23 in Sequence Listing and the amino acid sequence of the protein encoded by this gene, which is estimated on the basis of this nucleotide sequence, is the one represented by SEQ ID No. 24 in Sequence Listing. As the result of the homology search with the scaur2^S gene and the protein encoded thereby, it has been found out that cystic fibrosis transmembrane conductance regulator (CFTR) of mammals alone has a homology as low as 31%. Compared with this CFTR, however, the part having a high homology is limited to the region around the domain of the nucleotide binding. It is therefore obvious that the protein encoded by the scaur2^S gene is a protein which is completely different from CFTR in function and has never been known hitherto.

In order to prove the importance of the aur1 gene in the growth of cells, genes for disrupting the aur1 as shown in Fig. 4 and Fig. 5, in which genes coding for orotidine-5'-phosphate decarboxylase (ura4$^+$ in the case of *Schizo. pombe*, while URA3 in the case of *S. cerevisiae*) have been introduced midway in the aur1 gene, are prepared. When these aur1 disrupted genes are introduced into *Schizo. pombe* and *S. cerevisiae* respectively, the cells having the aur1 disrupted genes cannot grow at all. Thus it has been revealed that these genes and the proteins encoded thereby are essentially required for the growth of the yeast cells.

As the above examples clearly show, a gene regulating aureobasidin sensitivity can be isolated by using a organism having sensitivity to aureobasidin as a starting material and by carrying out the cloning with the use of various mutagenesis methods and/or screening methods depending on the organisms or the methods. Also, genes being hybridizable with the above-mentioned genes are involved in the scope of the first invention of the present invention. A gene regulating

aureobasidin sensitivity can be isolated by the following method. The genomic DNA library of an organism having sensitivity to aureobasidin is integrated into, for example, a high-expression vector of a yeast and transformed into the yeast. Then a clone having aureobasidin resistance is selected from the transformants and DNA is recovered from this clone. Thus the resistant gene can be obtained. As a matter of course, genes obtained by modifying some part of the gene regulating aureobasidin sensitivity thus obtained by some chemical or physical methods are involved in the scope of the first invention of the present invention.

The second invention of the present invention relates to a process for cloning a gene regulating aureobasidin sensitivity which is characterized by using the gene regulating aureobasidin sensitivity of the first invention of the present invention or a part thereof as a probe. Namely, by screening, by the hybridization method or the polymerase chain reaction (PCR) method with the use of a part (consisting of at least 15 oligonucleotides) or the whole of the gene as obtained above, a gene coding for a protein having a similar function can be isolated.

For example, a pair of primers of SEQ ID No. 25 and SEQ ID No. 26 in Sequence Listing are synthesized on the basis of the DNA nucleotide sequence of the spaur1^{R} gene represented by SEQ ID No. 15. Then PCR is performed by using cDNA of *C. albicans*, which is a pathogenic fungus, as a template with the use of the above-mentioned primers. The PCR is carried out and the PCR products are electrophoresed on an agarose gel and stained with ethidium bromide. In Fig. 6, the lanes 1, 2 and 3 show the results obtained by using cDNA of *C. albicans*, cDNA of *S. cerevisiae* and cDNA of *Schizo. pombe* as a template, respectively. As shown in Fig.6, a certain DNA fragment is specifically amplified.

By screening the genomic DNA library of *C. albicans* with the use of this DNA fragment as a probe, a DNA molecule having a gene (caaur1), which has the same function as that of the spaur1 and scaur1 genes and having the restriction enzyme map of Fig. 7 is obtained. The nucleotide sequence of this caaur1 gene is the one represented by SEQ ID No. 27 in Sequence Listing and the amino acid sequence of the protein encoded by this gene, which has been estimated on the basis of the above nucleotide sequence, is the one represented by SEQ ID No. 28 in Sequence Listing. It has a high homology with the proteins encoded by the spaur1 and scaur1 genes.

By screening the genomic DNA library of *C. albicans* with the use of a DNA fragment comprising the whole length or a part of the scaur2^S gene represented by SEQ ID No. 23 in Sequence Listing as a probe, a DNA fragment containing gene (caaur2), which has the same function as that of the scaur2 gene, and having the restriction enzyme map of Fig. 8 is obtained. The nucleotide sequence of a part of this caaur2 gene is the one represented by SEQ ID No. 29 in Sequence Listing and the amino acid sequence of the region encoded by this gene, which has been estimated on the basis of this nucleotide sequence, is the one represented by SEQ ID No. 30 in Sequence Listing. It has a high homology with the corresponding region of the protein encoded by the scaur2 gene.

The third invention of the present invention relates to an oligonucleotide comprising 15 or more bases which serves as the above-mentioned nucleic acid probe and is hybridizable with the gene regulating aureobasidin sensitivity, for example, the DNA fragment having the restriction enzyme map as shown in Fig. 1, Fig. 2 or Fig. 3. This nucleic acid probe is usable in, for example, the hybridization in situ, the identification of a tissue wherein the above-mentioned gene can be expressed, and the confirmation of the presence of a gene or mRNA in various vital tissues. This nucleic acid probe can be prepared by ligating the above-mentioned gene or a gene fragment to an appropriate vector, introducing it into a bacterium, allowing it to replicate in the bacterium, extracting from a disrupted cell suspension, cleaving with a restriction enzyme capable of recognizing the vector-ligating site, electrophoresing and then excising from the gel. Alternatively, this nucleic acid probe can be constructed by the chemical synthesis with the use of a DNA synthesizer or gene amplification techniques by PCR on the basis of the nucleotide sequence of SEQ ID Nos. 15, 17, 19, 21, 23, 27, 29 or 35 in Sequence Listing. This nucleic acid probe can be labeled with a radioisotope or a fluorescent substance to thereby elevate the detection sensitivity during use.

The fourth invention of the present invention relates to an antisense DNA of the above-mentioned gene regulating aureobasidin sensitivity, while the fifth invention of the present invention relates to an antisense RNA thereof. The introduction of the antisense DNA or antisense RNA into cells makes it possible to control the expression of the gene regulating aureobasidin sensitivity.

As examples of the antisense DNA to be introduced, antisense DNAs corresponding to the

genes regulating aureobasidin sensitivity of SEQ ID Nos. 15, 17, 19, 21, 23, 27, 29 or 35 in Sequence Listing and some parts thereof may be cited. SEQ ID No. 31 in Sequence Listing shows an example of this antisense DNA. It represents the sequence of an antisense DNA of the gene regulating aureobasidin sensitivity of SEQ ID No. 15 in Sequence Listing. A fragment obtained by appropriately cleaving some part of such an antisense DNA, and a DNA synthesized depending on such an antisense DNA sequence may be used as the antisense DNA.

As examples of the antisense RNA to be introduced, antisense RNAs corresponding to the genes regulating aureobasidin sensitivity of SEQ ID Nos. 15, 17, 19, 21, 23, 27, 29 or 35 in Sequence Listing and some parts thereof may be cited. SEQ ID No. 32 in Sequence Listing shows an example of this antisense RNA. It represents the sequence of an antisense RNA of the gene regulating aureobasidin sensitivity of SEQ ID No. 15 in Sequence Listing. A fragment obtained by appropriately cleaving some part of such an antisense RNA, an RNA synthesized depending on such an antisense RNA sequence, and an RNA prepared with RNA polymerase in an *in vitro* transcription system by using the DNA corresponding to the gene regulating aureobasidin sensitivity of SEQ ID No. 15 or SEQ ID No. 17 in Sequence Listing or a part thereof may be used as the antisense RNA.

These antisense DNA and antisense RNA may be chemically modified so as to prevent degradation *in vivo* or to facilitate passage through a cell membrane. A substance capable of inactivating mRNA, for example, ribozyme may be linked thereto. The antisense DNA and antisense RNA thus prepared are usable in the treatment of various diseases such as mycoses accompanied by an increase in the amount of mRNA coding for a protein regulating aureobasidin sensitivity.

The sixth invention of the present invention relates to a recombinant plasmid having a gene coding for a protein regulating aureobasidin sensitivity being integrated into an appropriate vector. For example, a plasmid, in which a gene regulating aureobasidin sensitivity gene has been integrated into an appropriate yeast vector, is highly useful as a selection marker gene, since a transformant can be easily selected thereby with the guidance of the chemical resistance by using aureobasidin.

Also, the recombinant plasmid can be stably carried by, for example, *Escherichia coli*. Examples of vectors which are usable in this case include pUC118, pWH5, pAU-PS, Traplexll9 and pTV118. pAU-PS having the spaur1^S gene integrated therein is named pSPAR1. pWH5 having

the spaur1^S gene integrated therein is named pSCAR1. pWH5 having the scaur2^R gene integrated therein is named pSCAR^2. Traplexll9 vector having the caaur1 gene integrated therein is named pCAAR1. pTV118 vector having a part of the caaur2 gene integrated therein is named pCAAR2N. Each of these recombinant plasmids is transformed into *E. coli*. It is also possible to express these plasmids in an appropriate host. Such a gene is reduced exclusively into the open reading frame (ORF) to be translated into a protein by cleaving with an appropriate restriction enzyme, if necessary, and then bound to an appropriate vector. Thus an expression recombinant plasmid can be obtained. When *E. coli* is used as the host, plasmids such as pTV118 may be used as a vector for the expression plasmid. When a yeast is used as the host, plasmids such as pYES2 may be used as the vector. When mammalian cells are used as the host, plasmids such as pMAMneo may be used as the vector.

The seventh invention of the present invention relates to a transformant having the above-mentioned recombinant plasmid which has been introduced into an appropriate host. As the host, *E. coli*, yeasts and mammalian cells are usable. *E. coli* JM109 transformed by pSPAR1 having the spaur1^S gene integrated therein has been named and designated as *Escherichia coli* JM109/pSPAR1 and deposited at National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (1-3, Higashi 1 chome Tsukuba-shi Ibaraki-ken 305, JAPAN), in accordance with the Budapest Treaty under the accession number FERM BP-4485. *E. coli* HB101 transformed by pSCAR1 having the scaur1^S gene integrated therein has been named and designated as *Escherichia coli* HB101/pSCAR1 and deposited at National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology in accordance with the Budapest Treaty under the accession number FERM BP-4483. *E. coli* HB101 transformed by pSCAR2 having the scaur2^R gene integrated therein has been named and designated as *Escherichia coli* HB101/pSCAR2 and deposited at National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology in accordance with the Budapest Treaty under the accession number FERM BP-4484. *E. coli* HB101 transformed by pCAAR1 having the caaurl^S gene integrated therein has been named and designated as *Escherichia coli* HB101/pCAAR1 and deposited at National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology in accordance with the Budapest Treaty under the accession number FERM

BP-4482. *E. coli* HB101 transformed by pCAAR2N having a part of the caaur2 gene integrated therein has been named and designated as *Escherichia coli* HB101/pCAAR2N and deposited at National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology in accordance with the Budapest Treaty under the accession number FERM BP-4481.

A transformant capable of expressing a protein regulating aureobasidin sensitivity can be obtained by transforming a expression recombinant plasmid into an appropriate host, as described above. For example, a yeast having a recombinant plasmid as shown in Fig. 9 introduced thereinto is usable for this purpose.

The eighth invention of the present invention relates to a process for producing a protein regulating aureobasidin sensitivity which comprises incubating a transformant according to the sixth invention of the present invention, which contains a gene coding for this protein, in an appropriate nutritional medium, allowing the expression of the protein, then recovering the protein from the cells or the medium and purifying the same. For the expression of the gene coding for this protein, *E. coli*, a yeast or mammalian cells are employed as a host. When the yeast having the recombinant plasmid of Fig. 9 is incubated in a medium containing galactose, for example, the protein regulating aureobasidin sensitivity which is encoded by the scaur1^S gene can be expressed.

The ninth invention of the present invention relates to an isolated protein regulating aureobasidin sensitivity. As examples of such a protein, those encoded by the above-mentioned spaur1, scaur1, scaur2, caaur1 and caaur2 genes can be cited.

The spaur1^S gene codes for a protein having an amino acid sequence represented by SEQ ID No. 18 in Sequence Listing, while the scaur1^S gene codes for a protein having an amino acid sequence represented by SEQ ID No. 22 in Sequence Listing. By the northern hybridization with the use of a DNA fragment of the spaur1 gene as a probe, mRNAs are detected from a sensitive strain (Fig. 10). Thus the expression of the spaur1 gene is confirmed.

Fig. 10 is an autoradiogram showing the results of the northern hybridization wherein mRNAs obtained from cells of a sensitive strain of *Schizo. pombe* in the logarithmic growth phase (lane 1), cells of a resistant strain in the logarithmic growth phase (lane 2), cells of the sensitive strain in the stationary phase (lane 3) and cells of the resistant strain in the stationary phase (lane 4) are electrophoresed on a 1.2% agarose gel containing formaldehyde.

The tenth invention of the present invention relates to an antibody against the above-mentioned protein regulating aureobasidin sensitivity. For example proteins having amino acid sequences of SEQ ID Nos. 16, 18, 20, 22, 24, 28, 30 or 36 in Sequence Listing and peptides comprising some parts of these amino acid sequences may be used as an antigen. The former antigens can be prepared through the expression in a transformant followed by purification, while the latter antigens can be synthesized on, for example, a marketed synthesizer. The antibody is produced by the conventional method. For example, an animal such as a rabbit is immunized with the above-mentioned protein or a peptide fragment together with an adjuvant to thereby give a polyclonal antibody. A monoclonal antibody can be produced by fusing antibody-producing B cells, which have been obtained by immunizing with an antigen, with myeloma cells, screening hybridomas producing the target antibody, and incubating these cells. As will be described hereinafter, these antibodies are usable in the treatment and diagnosis for animal and human diseases in which the above-mentioned proteins participate, such as mycoses.

For example, a peptide corresponding to the part of the 103- to 113-positions in the amino acid sequence of SEQ ID No. 22 is synthesized on a synthesizer and then bound to a carrier protein. Then a rabbit is immunized therewith and thus a polyclonal antibody is obtained. In the present invention, keyhole limpet hemocyanin (KLH) is used as the carrier protein. Alternatively, bovine serum albumin and ovalbumin are usable therefor.

The eleventh invention of the present invention relates to a process for detecting a protein regulating aureobasidin sensitivity by using the above-mentioned antibody. The detection can be carried out by detecting the binding of the antibody to the protein or measuring the amount of binding. For example, the protein or the cells producing the same can be detected by treating with a fluorescence-labeled antibody and then observing under a fluorescence microscope. The amount of the antibody bound to the protein can be measured by various known methods. For example, *S. cerevisiae* cells are stained by the immunofluorescent antibody technique by using the above-mentioned antibody and a secondary antibody such as FITC-labeled anti-rabbit antibody. Thus it is clarified that the protein encoded by the scaur1 gene is distributed all over the cells. Further, a yeast having the recombinant plasmid of Fig. 9 introduced thereinto is incubated in a medium containing glucose or galactose. The cells thus obtained are disrupted with glass beads and

proteins are solubilized. Then these proteins are separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and the western blotting is carried out in the conventional manner by using the above-mentioned polyclonal antibody and peroxidase-labeled anti-rabbit antibody. Consequently, the protein encoded by the scaur1 gene can be detected, as Fig. 11 shows.

Fig. 11 shows the results of the western blotting wherein the proteins prepared from the cells obtained by the incubation in the presence of glucose (lane 1) or galactose (lane 2) are subjected to SDS-PAGE. A main band binding to the polyclonal antibody of the present invention is detected at around 38 kDa.

The twelfth invention of the present invention relates to a process for detecting a gene regulating aureobasidin sensitivity, for example, mRNA at the expression of a protein, by using the above-mentioned oligonucleotide as a nucleic acid probe. This process is applicable to the diagnosis for various diseases, including mycoses, associated with an abnormal amount of mRNA coding for the protein. For example, nucleic acids are precipitated from disrupted cells and mRNA is hybridized with a radioisotope-labeled nucleic acid probe on a nitrocellulose membrane. The amount of binding can be measured by autoradiography (Fig. 10) or with a scintillation counter.

The thirteenth invention of the present invention relates to a process for efficient screening of a novel antimycotic by using the transformant of the seventh invention of the present invention or the protein regulating aureobasidin sensitivity of the ninth invention of the present invention. For example, a drug exerting its effect on the protein or the gene of the present invention can be efficiently found out through a comparison of the activity on a transformant containing a sensitive gene with the activity on a transformant containing a resistant gene or a comparison between the activities on transformants differing in expression level from each other. Also, the screening can be efficiently carried out by measuring the affinity for the protein of the present invention, for example, the activity of inhibiting the binding of radiolabeled-aureobasidin to the protein.

As the above-mentioned examples clearly show, a gene regulating the aureobasidin sensitivity corresponding to each organism or each method can be isolated by employing a starting material, which is an organism having the sensitivity to aureobasidin, and effecting cloning by conducting various mutagenesis and/or screening treatments in the same manner as the one described above. Moreover, genes hybridizable with these genes can be isolated. As a matter of

course, it is possible to prepare modified genes by partly altering the genes regulating the aureobasidin sensitivity obtained above by chemical, physical or genetic engineering techniques.

In the present invention, an aureobasidin resistant gene refers to a gene which is capable of imparting the resistance to an antimycotic aureobasidin when integrated into a host fungus. This gene codes for a protein imparting an aureobasidin resistance.

The aureobasidin resistant gene is exemplified typically by the above-mentioned spaur1^R and scaur1^R. Such a gene acts predominantly and the resistance conferred by this gene is selective to aureobasidin. That is to say, it does not cause any substantial change in the sensitivity to other drugs.

The aureobasidin resistant gene also involves genes which are hybridizable with spaur1^R and scaur1^R and impart the aureobasidin resistance to a host fungus (for example, genes prepared by partly altering the spaur1^R or scaur1^R gene by chemical, enzymatic, physical or genetic engineering techniques).

Furthermore, the aureobasidin resistant gene involves a gene coding for a protein, which has an amino acid sequence obtained by subjecting a protein (Aur1^{R}p) capable of imparting the aureobasidin resistance to at least one modification selected from replacement, insertion and deletion of amino acid residue(s) and shows the activity of imparting the aureobasidin resistance.

The replacement, insertion and deletion of amino acid residue(s) from Aur1^{R}p can be effected by a site-specific mutagenesis. A DNA coding for the isolated Aur1^{R}p or a DNA coding for the protein capable of imparting the aureobasidin sensitivity (Aur1^{S}p) can be easily modified by effecting at least one of the replacement, insertion and deletion of nucleotide(s) and thus a novel DNA coding for a mutant of Aur1^{R}p can be obtained. Regarding the replacement, insertion and deletion of amino acid residue(s), the conversion of the amino acid(s) is based on one which can be effected by genetic engineering techniques without deteriorating the biological activity. In order to appropriately effect the mutation on the residue at a specific site, the target codon is subjected to random mutagenesis and a mutant having the desired activity is screened from the ones thus expressed. The mutant obtained by insertion involves a fused protein wherein Aur1^{R}p or its fragment is bound to another protein or polypeptide at the amino terminal and/or the carboxyl terminal of the Aur1^{R}p or its fragment via a peptide bond. In order to delete amino acid residue(s),

it is also possible to replace an arbitrary amino acid codon in the amino acid sequence with a termination codon by the gapped duplex method to thereby delete the region on the carboxyl terminal side of the replaced amino acid residue from the amino acid sequence. Alternatively, a DNA coding for a protein, from which the amino terminal and/or carboxyl terminal regions in an arbitrary length have been deleted, can be obtained by the deletion method comprising degrading the coding DNA from the region(s) corresponding to the amino terminal and/or the carboxyl terminal of the amino acid sequence [Gene, 33, 103 - 119 (1985)] or a PCR method with the use of primers containing an initiation codon and/or a termination codon. Known examples of the site-specific mutagenesis method include the gapped duplex method with the use of oligonucleotide(s) [Methods in Enzymology, 154, 350 - 367 (1987)], the uracil DNA method with the use of oligonucleotide(s) [Methods in Enzymology, 154, 367 - 382 (1987)], the nitrous acid mutation method [Proc. Natl. Acad. Sci. USA, 79, 7258 - 7262 (1982)] and the cassette mutation method [Gene, 34, 315 - 323 (1985)].

The present inventors have found out that Aur1^{S}p represented by SEQ ID No. 22 in the Sequence Listing can be converted into Aur1^{R}p by replacing the 240th residue Ala by another amino acid residue, thus completing the seventeenth and eighteenth inventions.

The Aur1^{R}p of the seventeenth invention is one wherein the 240th residue Ala of Aur1^{S}p represented by SEQ ID No. 22 in the Sequence Listing has been replaced by another amino acid residue. Other amino acid residues may be replaced, inserted or deleted by using chemical, physical or genetic engineering techniques, so long as the biological activity is not deteriorated thereby. The Aur1^{R}p of the seventeenth invention may be appropriately prepared through genetic engineering techniques by using a DNA coding for Aur1^{S}p represented by SEQ ID No. 47 in the Sequence Listing. Its biological activity can be assayed by measuring the activity of converting aureobasidin sensitive cells into resistant cells. The Aur1^{R}p of the seventeenth invention is one having an enhanced activity of converting aureobasidin sensitive cells into resistant cells compared with Aur1^{R}p represented by SEQ ID No. 20 in the Sequence Listing.

A preferable Aur1^{R}p is one having an enhanced activity of converting aureobasidin sensitive cells into resistant cells compared with Aur1^{R}p represented by SEQ ID No. 20 in the Sequence Listing. A DNA coding for this Aur1^{R}p can be appropriately used in the present invention.

In an example of particularly preferable embodiment of Aur1^{R}p, a mutant can be obtained by replacing the 240th residue Ala by Cys. The amino acid sequence of an example of such a mutant is shown in SEQ ID No. 42 in the Sequence Listing. This mutant is referred to as Aur1^{R}p (A240C). It is also possible to obtain a mutant wherein the 158th residue Phe and the 240th residue Ala of Aur1^{S}p have been replaced respectively by Tyr and Cys. The amino acid sequence of this mutant is shown in SEQ ID No. 43 in the Sequence Listing. This mutant is referred to as Aur1^{R}p (F158Y, A240C). Each of these mutants has a stronger ability to impart aureobasidin resistance than that of the protein represented by SEQ ID No. 40 in the Sequence Listing [Aur1^{R}p (F158Y)] wherein the 158th residue Phe of Aur1^{S}p has been replaced by Tyr.

The aureobasidin resistant gene to be used in the present invention is exemplified by the DNAs represented by SEQ ID Nos. 44 to 46 in the Sequence Listing. The DNA represented by SEQ ID No. 46 in the Sequence Listing is one coding for Aur1^{R}p (F158Y), the DNA represented by SEQ ID No. 44 in the Sequence Listing is one coding for Aur1^{R}p (A240C), and the DNA represented by SEQ ID No. 45 in the Sequence Listing is one coding for Aur1^{R}p (F158Y, A240C).

A replication plasmid can be prepared by integrating a gene, which coded for a protein regulating the aureobasidin sensitivity, into an appropriate vector. For example, a plasmid prepared by integrating an aureobasidin resistant gene into an appropriated yeast vector is highly useful as a selective marker gene, since a transformant can be easily selected thereby depending on the drug resistance with the use of aureobasidin. As the vector for yeasts, use can be made of ones of YR_p, YC_p, YE_p and YI_p types.

Also, the replication plasmid can be stably carried by, for example, *Escherichia coli*, as described above. Examples of vectors which are usable in this case include pUC118, pWH5, pAU-PS, Traplex119 and pTV118.

The integration vector containing the aureobasidin resistant gene of the present invention is a linear vector which can be usually prepared by cleaving a replication plasmid containing the aureobasidin resistant gene into a linear form. The cleavage point in the replication plasmid will be described hereinbelow.

Fig. 13 shows a process wherein an aureobasidin resistant gene in a chromosome integration vector undergoes homologous recombination with the host chromosome being homologous

therewith (i.e., an aureobasidin sensitive gene) and thus aureobasidin sensitive cells are converted into aureobasidin resistant cells. A replication plasmid containing the aureobasidin resistant gene is cleaved into a linear form at one position in the aureobasidin resistant gene sequence with an appropriate restriction enzyme. The vector thus linearized undergoes homologous recombination with the aureobasidin sensitive gene in the host chromosome being homologous therewith. Thus the aureobasidin resistance is imparted to the host cells. When the replication plasmid contains a foreign gene, then the aureobasidin resistance and the foreign gene are imparted to the host cells. For example, a replication vector pAUR1aare for preparing a linear vector, which contains scaur1^R and human acylamino acid releasing enzyme (AARE) described in Japanese Patent Laid-Open No. 254680/1991, is prepared. *Escherichia coli* JM109 strain having this vector introduced therein was named and indicated as *Escherichia coli* JM109/pAUR1aare and has been deposited at National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology under the accession number FERM P-14366. To linearize a replication vector, use can be effectively made of a restriction enzyme cleavage site which exists not in the target foreign gene moiety but in the aureobasidin resistant gene. In the cases of, for example, scaur1^R originating in *S. cerevisiae* and spaur1^R originating in *Schizo. pombe*, restriction enzyme sites of StuI, etc. and BalI, etc. are usable respectively.

In a preferable form, the vector of the present invention may contain an aureobasidin resistant gene and a foreign gene and other genes originating in replication vectors may be eliminated therefrom. Promoters, terminators, etc. for expressing the aureobasidin resistant gene and the foreign gene may be selected depending on the characters of the host. As a matter of course, the promoter parts of the DNAs represented by SEQ ID Nos. 15 and 19 in the Sequence Listing can be used as a promoter for expressing the function of the aureobasidin resistant gene. In the case of *S. cerevisiae*, use can be made of, for example, promoters of alcohol dehydrogenase gene (ADH1) and glyceraldehyde-3-phosphate dehydrogenase gene (GPD) and the terminator of cytochrome C1 gene (CYC1). These promoters and terminators may be different from those for expressing the aureobasidin resistant gene.

In the present invention, the term "foreign gene" refers to a gene which is foreign to the host fungal cells, i.e., an alien gene. Examples thereof include a nonfungal gene, a modified gene, a gene

of a fungal species different from the host and a self-cloned gene. More particularly, genes participating in fermentation, alcohol resistance, saccharification and the formation of taste components or aroma components fall within this category.

The fifteenth invention relates to a process for producing an aureobasidin resistant transformant. An aureobasidin resistant transformant can be created by, for example, preparing a replication vector containing the above-mentioned aureobasidin resistant gene, cleaving it at one position in the aureobasidin resistant gene in the replication vector to give a linear chromosome integration vector for a host fungus, adding this vector to aureobasidin sensitive host fungal cells under such conditions as to allow the transformation of the fungal cells, thus integrating the vector into the host chromosome, incubating the transformant in a medium suitable for the proliferation of the host cells containing the antibiotic aureobasidin, and screening the aureobasidin resistant transformant thus proliferating. The transformation may be effected in accordance with publicly known methods such as the protoplast generation procedure, the lithium acetate procedure or the electroporation procedure. The medium to be used herein is not particularly restricted, so long as it is usable in the proliferation of fungi. Examples of such a medium commonly employed include Sabouraud's dextrose medium, a YPD medium, a czapek medium and a YNBG medium. The concentration of the aureobasidin added varies depending on the host fungal cells having the sensitivity and usually ranges from 0.05 to 80 μg/ml.

The transformant of the sixteenth invention can be obtained by the process of the fifteenth invention.

As an example of the transformant according to the present invention, Sake yeast Kyokai K-701 having scaur1^R and AARE gene integrated into the chromosome has been deposited at National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology under the accession number FERM P-1437. The transformant thus obtained by using the chromosome integration vector of the present invention has an aureobasidin resistance imparted thereto and the foreign gene integrated thereinto which is held on the chromosome in a stable state. These characteristics make it highly useful in industrial uses, etc.

The Aur1^{R}p of the seventeenth invention can impart the aureobasidin resistance to monoploid yeasts and diploid yeasts, in particular, practically usable ones. Namely, it is highly

useful in breeding *S. cerevisiae* which has been widely applied to liquors such as sake, shochu, beer and wine and fermented foods such as bread. Further, the Aur1^{R}p of the seventeenth invention is applicable to fungi other than *S. cerevisiae* and useful in, for example, breeding and genetic engineering application of other fungi.

For example, the Aur1^{R}p of the seventeenth invention is capable of imparting the aureobasidin resistance to *C. albicans*. A vector having the DNA coding for this Aur1^{R}p is the first vector for genetic engineering uses provided for *C. albicans*.

It is known that *C. albicans* is a fungus causative of mycosis. With the recent increase in opportunistic infection, it has been needed to conduct studies for clarifying the causes of the pathogenicity. The Aur1^{R}p of the seventeenth invention and the above-mentioned vector are highly useful in genetic studies on *C. albicans*.

The present inventors have further found out that molds such as *Aspergillus nidulans* (hereinafter referred to simply as *A. nidulans*) and *Aspergillus fumigatus* (hereinafter referred to simply as *A. fumigatus*) are sensitive to aureobasidin. Thus we have mutated sensitive cells of *A. nidulans* into resistant cells and succeeded in the isolation of a gene capable of imparting the resistance to aureobasidin (a resistant gene) from the corresponding resistant cells. Further, we have disclosed the existence of a protein encoded by this gene. We have also successfully found novel genes regulating aureobasidin sensitivity from aureobasidin sensitive *A. nidulans* and *A. fumigatus* by using a DNA fragment of the above-mentioned gene as a probe. Furthermore, we have found out that the detection of this gene enables the diagnosis of diseases caused by these cells (for example, mycosis caused by fungi) and that the antisense DNA or antisense RNA, which inhibits the expression of the gene regulating aureobasidin sensitivity characteristic of the cells, is usable as a remedy for diseases caused by these cells (for example, an antimycotic for mycosis).

The term "a protein regulating aureobasidin sensitivity" as used herein means a protein which is contained in an organism, in particlar a mold, showing a sensitivity to aureobasidin. This protein is required for achieving a sensitivity or resistance to aureobasidin. The term "a gene regulating aureobasidin sensitivity" means a gene which encodes such a protein regulating aureobasidin sensitivity and a sensitive gene and a resistant gene fall within this category. The aureobasidin sensitivity of an organism varies depending on the molecular structure or amount of such a protein

or gene regulating aureobasidin sensitivity carried by the organism.

The term "a functional derivative of the protein or gene regulating aureobasidin sensitivity" as used herein means one which has a biological activity substantially comparable to that of the protein or DNA regulating aureobasidin sensitivity. It include fragments, variants, mutants, analogs, homologs and chemical derivatives. A variant means one which is substantially analogous to the whole protein or a fragment originating therein in structure and/or function. That is to say, one molecule which is essentially analogous to another in activity is regarded as a mutant, even though these two molecules are different in molecular structure or amino acid sequence from each other. The functional derivatives include proteins showing an amino acid sequence with at least one modification selected from among replacement, insertion and deletion of amino acid residue(s) and having a comparable biological activity and genes encoding these. The protein regulating aureobasidin sensitivity may be subjected to the replacement, insertion and deletion of amino acid residues by a site-specific mutagenesis. The isolated DNA encoding the protein regulating aureobasidin sensitivity can be easily subjected to at least one modification selected from among replacement, insertion and deletion of nucleotides and thus a novel DNA encoding the protein regulating aureobasidin sensitivity and its functional derivatives can be obtained.

Regarding the replacement, insertion and deletion of amino acid residues, one or more amino acids can be converted by genetic engineering techniques and those suffering from no injury to the biological activity should be selected. To properly effect a mutation on the residue at a specified site, mutagenesis is performed at random on the target codon and a mutant having the desired activity is screened from the ones thus expressed. The mutant obtained by insertion involves a fused protein wherein the protein regulating aureobasidin sensitivity or its functional derivative or a fragment thereof is bound via a peptide bond to another protein or polypeptide at the amino terminal and/or the carboxy terminal of the protein regulating aureobasidin sensitivity or its functional derivative or a fragment thereof. To delete amino acid residue(s), an arbitrary amino acid codon in the amino acid sequence may be replaced by a termination codon by the site-specific mutagenesis. Thus the region on the carboxy terminal side of the replaced amino acid residue can be deleted from the amino acid sequence. Alternatively a DNA coding for a protein, from which the amino terminal and/or carboxy terminal regions in an arbitral length have been deleted, can be obtained by the

deletion method comprising degrading a coding DNA from the region(s) corresponding to the amino terminal and/or the carboxy terminal of the amino acid sequence [Gene, 33, 103-119 (1985)] or a PCR method with the use of primers containing an initiation codon and/or a termination codon. Known examples of the site-specific mutagenesis method include the gapped duplex method with the use of oligonucleotide(s) [Methods in Enzymology, 154, 350-367 (1987)], the uracil DNA method with the use of oligonucleotide(s) [Methods in Enzymology, 154, 367-382 (1987)], the nitrous acid mutation method [Proc. Natl. Acad. Sci. USA, 79, 7258-7262 (1982)] and the cassette mutation method [Gene, 34, 315-323 (1985)].

The nineteenth invention relates to a gene regulating aureobasidin sensitivity obtained from a mold exemplified by one belonging to the genus *Aspergillus* or its functional derivative. In order to isolate this gene, aureobasidin sensitive cells are first subjected to a mutagenesis to thereby derive a resistant strain therefrom. Then a DNA library is prepared from the chromosome DNAs or cDNAs of this resistant strain and a gene capable of imparting the resistance (a resistant gene) is cloned from this library. Similarly, a DNA library of a sensitive strain is prepared and DNA molecules hybridizable with the resistant gene are isolated and cloned. Thus a sensitive gene can be isolated.

The mutagenesis is performed by, for example, treating with a chemical such as ethylmethane sulfonate (EMS) or N-methyl-N'-nitro-N-nitro-soguanidine (NTG) or by ultraviolet or other radiation. A mutant that has acquired the resistance can be screened by culturing the mutagenized cells in a nutritional medium containing aureobasidin at an appropriate concentration under appropriate conditions. The resistant strain thus obtained may vary depending on the method and conditions selected for the mutagenesis. It is further possible to select strains differing in the extent of resistance by varying the aureobasidin concentration at the screening. It is also possible to select a temperature-sensitive resistant strain by varying the temperature at the screening. Since there are two or more mechanisms of the resistance to aureobasidin, two or more resistant genes can be isolated by genetically classifying these resistant strains.

The genes regulating aureobasidin sensitivity of molds belonging to the genus Aspergillus of the present invention include a gene anaurl^R isolated from a resistant mutant of *A. nidulans,* a gene anaurl^S isolated from a sensitive strain of *A. nidulans* and a gene afaurl^S isolated from a

sensitive strain of *A. fumigatus*.

The attached Fig. 15 shows the restriction enzyme map of the genomic DNA of the gene anaurl^R regulating aureobasidin sensitivity and originating in a mold of *Aspergillus,* Fig. 16 shows the restriction enzyme map of the cDNA of the gene anaurl^S and Fig. 17 shows the restriction enzyme map of the cDNA of the gene afaurl^S.

A. nidulans sensitive to aureobasidin is mutagenized by UV irradiation and a genomic library of the resistant strain thus obtained is prepared. From this library, a DNA fragment containing a resistant gene (anaurl^R) and having the restriction enzyme map of Fig. 15 is isolated. This gene has a DNA sequence represented by SEQ ID NO. 1 in the Sequence Listing. The amino acid sequence of a protein encoded by this gene, which is estimated on the basis of this DNA sequence, is the one represented by SEQ ID NO. 2 in the Sequence Listing. By the hybridization with the use of this resistant gene, a cDNA fragment containing a sensitive gene (anaurl^S) and having the restriction enzyme map of Fig. 16 is isolated from a cDNA library of a sensitive strain. This sensitive gene has a DNA sequence represented by SEQ ID NO. 3 in the Sequence Listing. The amino acid sequence of a protein encoded by this gene, which is estimated on the basis of this base sequence, is the one represented by SEQ ID NO. 4 in the Sequence Listing. A comparison between the sequences of SEQ ID NO. 3 and SEQ ID NO. 1 reveals that the genomic DNA has one intron (intervening sequence) ranging from the base at the position 1508 to the one at the position 1563 in SEQ ID NO. 1. Further, G at the position 1965 in SEQ ID NO. 1 has been mutated into T. A comparison between the sequences of SEQ ID NO. 4 and SEQ ID NO. 2 reveals that the amino acid glycine at the position 275 has been mutated into valine at the amino acid level, thus giving the resistance. The nineteenth invention also involves genes constructed by chemically or physically altering a part of the genes of the present invention which regulate aureobasidin sensitivity and originate in molds.

The twentieth invention relates to a method for cloning a gene regulating aureobasidin sensitivity and originating in a mold such as one of the genus *Aspergillus* or its functional derivative. This method comprises using the gene of the nineteenth invention regulating aureobasidin sensitivity and originating in a mold, its functional derivative, or a part of the same as a probe. That is to say, a gene encoding a protein having a comparable function can be isolated by the hybridization method or the polymerase chain reaction (PCR) method with the use of the whole or a part of the gene

(consisting of at least 15 oligonucleotides) obtained above as a probe.

To examine a region appropriately usable as the above-mentioned probe, the present inventors have compared the amino acid sequence of the protein encoded by the gene anaurl^S of the present invention (SEQ ID NO. 4 in the Sequence Listing) and the amino acid sequence of the protein encoded by the gene afaurl^S of the present invention (SEQ ID NO. 5 in the Sequence Listing) with the amino acid sequence of the protein encoded by an aureobasidin sensitive gene (scaurl^S) originating in *S. cerevisiae* (SEQ ID NO. 6), the amino acid sequence of the protein encoded by another aureobasidin sensitive gene (spaurl^S originating in *Schizo. pombe* (SEQ ID NO. 7) and the amino acid sequence of the protein encoded by a gene regulating aureobasidin sensitivity (caaurl) originating in *C. albicans* (SEQ ID NO. 8), each described in Canadian Patent No. 2124034. As a result, no homology is observed as the whole. However, it has been revealed for the first time that there is a characteristic sequence having been conserved in common in these heterogenous genes regulating aureobasidin sensitivity. This conversed sequence has been very well conserved (homology: 80% or above) and is composed of at least eight amino acid residues, which corresponds to a sufficiently long length to be used as a probe. Fig. 18 shows a comparison among the amino acid sequences represented by SEQ ID NOs. 4 to 8 wherein three sequences (Box-1 to Box-3) named "Box sequences" by the inventors correspond to the conserved sequence. Thus, a gene regulating aureobasidin sensitivity and originating in mold or its functional derivative can be cloned by using a primer or a probe constructed from the amino acid sequence of Box 1, 2 or 3 respectively represented by SEQ ID NOs. 9, 10 or 11 in the Sequence Listing.

The amino acid sequences given in five rows in Fig. 18 correspond respectively to SEQ ID NO. 4 (the top row), SEQ ID NO. 5 (the second row), SEQ ID NO. 6 (the third row), SEQ ID NO. 7 (the fourth row) and SEQ ID NO. 8 (the bottom row).

The target gene encoding the protein regulating aureobasidin sensitivity or its functional derivative may be obtained by hybridization in, for example, the following manner. First, chromosomal DNAs obtained from the target gene source or cDNAs constructed from mRNAs with the use of a reverse transcriptase are connected to a plasmid or a phage vector in accordance with the conventional method and introduced into a host to thereby prepare a library. After incubating this library on a plate, the colonies or plaques thus formed are transferred onto a nitrocellulose or

nylon membrane and the DNAs are denatured and thus immobilized on the membrane. This membrane is incubated in a solution containing a probe which has been preliminarily labeled with radio isotope ^{32}p, etc. (The probe to be used herein may be a gene encoding the amino acid sequence represented by SEQ ID NO. 4 in the Sequence Listing or a part of the same. For example, use can be made of the gene represented by SEQ ID NO. 3 in the Sequence Listing or a part of the same. It is appropriate to use therefor a base sequence which is composed of at least 15 bases and encodes one of the amino acid sequences represented by SEQ ID NOs. 9 to 11 in the Sequence Listing or a part of the same.) Thus DNA hybrids are formed between the DNAs on the membrane and the probe. For example, the membrane having the DNAs immobilized thereon is hybridized with the probe in a solution containing 6 x SSC, 1% of sodium lauryl sulfate, 100 μg/ml of salmon sperm DNA and 5 x Denhardt's solution (containing bovine serum albumin, polyvinylpyrolidone and Ficoll each at a concentration of 0.1%) at 65°C for 20 hours. After the completion of the hybridization, nonspecifically adsorbed matters are washed away and clones forming hybrids with the probe are identified by autoradiography, etc. Into the clone thus obtained, a gene encoding the target protein has been included.

It is confirmed whether or not the obtained gene is the one encoding the target protein regulating aureobasidin sensitivity or its functional derivative, after the DNA sequence of the obtained gene is identified by, for example, the following method.

A clone obtained by the hybridization may be sequenced in the following manner. When the recombinant all is *Escherichia coli,* it is incubated in a test tube, etc. and the plasmid is extracted by a conventional method. Then it is cleaved with restriction enzymes and an insert thus excised therefrom is subcloned into an M13 phage vector, etc. Next, the base sequence is identified by the dideoxy method. When the recombinant is a phage, the base sequence can be identified fundamentally by the same steps. These fundamental experimental procedures to be used from the cell culture to the DNA sequencing are described in, for example, Molecular Cloning, A Laboratory Manual, T. Maniatis et al., Cold Spring Harbor Laboratory Press (1982).

To confirm whether or not the obtained gene is the one encoding the target protein regulating aureobasidin sensitivity or its functional derivative, the amino acid sequence thus identified is compared with the amino acid sequence represented by SEQ ID No. 4 in the Sequence

Listing to thereby know the protein structure and amino acid sequence homology.

To examine whether or not the obtained gene sustains a sensitivity or resistance to aureobasidin, the obtained gene is transformed into sensitive cells and the aureobasidin sensitivity of the transformed cells thus obtained is determined to thereby reveal the activity of the gene. Alternatively, the activity can be determined by transforming the obtained gene into cells from which the activity has been eliminated by disrupting or mutating the gene regulating aureobasidin sensitivity. It is preferable that the above-mentioned gene to be transformed contains sequences required for the expression (promoter, terminator, etc.) in the upstream and/or downstream of the gene so as to enable the expression in the cells transformed.

When the obtained gene fails to contain the whole region encoding the protein regulating aureobasidin sensitivity or its functional derivative, the base sequence of the whole region encoding the protein regulating aureobasidin sensitivity or its functional derivative which is hybridizable with the gene of the present invention encoding the protein regulating aureobasidin sensitivity or its functional derivative can be obtained by preparing synthetic DNA primers on the basis of the gene thus obtained, amplifying the missing region by PCR or further screening a DNA library or a cDNA library with the use of a fragment of the obtained gene as a probe.

For example, a cDNA molecule having the restriction enzyme map of Fig. 17, which contains a gene (afaurl^s) of *A. fumigatus* being comparable in function to the gene anaurl^s, can be obtained by screening a cDNA library of a pathogenic fungus *A. fumigatus* with the use of a DNA fragment of the PstI-EcoRI fragment (921 bp) of Fig. 16 as a probe. This gene has a base sequence represented by SEQ ID NO. 12 in the Sequence Listing and the amino acid sequence of a protein encoded by this gene, which is estimated on the basis of this base sequence, is the one represented by SEQ ID NO. 5 in the Sequence Listing. When the genes anaurl^s and afaurl^s are compared, a homology of 87% is observed at the amino acid level. Further, genomic DNAs prepared from *Aspergillus niger* (hereinafter referred to simply as *A. niger*) and *Aspergillus oryzae* (hereinafter referred to simply as *A. oryzae*) are subjected to the Southern blotting analysis with the use of a DNA fragment of the gene anaurl^s as a probe. As a result, it is revealed that genes regulating aureobasidin sensitivity occur in *A. niger* and *A. oryzae*. It is also possible to isolate genes regulating aureobasidin sensitivity from molds other than those belonging to the genus *Aspergillus*, for

example, ones of the genus *Penicillium*.

The twenty-first invention relates to the above-mentioned nucleic acid probe, i.e., an oligonucleotide which is composed of at least 15 bases and hybridizable with a gene regulating aureobasidin sensitivity, for example, a DNA fragment having a restriction enzyme map of Fig. 15, 16 or 17.

This nucleic acid probe is applicable to *in situ* hybridization, the confirmation of a tissue wherein the above-mentioned gene is expressed, the confirmation of the existence of a gene or mRNA in various vital tissues, etc. This nucleic acid probe can be prepared by ligating the above-mentioned gene or its fragment to an appropriate vector, introducing it into a bacterium followed by replication, extracting with phenol, etc. from a disrupted cell solution, cleaving with restriction enzymes capable of recognizing the ligation site with the vector, electrophoresing and excising from the electrophoresis gels.

Alternatively, this nucleic acid probe can be prepared by a chemical synthesis with the use of a DNA synthesizer or gene amplification techniques by PCR on the basis of each of the base sequences represented by SEQ ID NOs. 1, 3 and 12 in the Sequence Listing. Examples of sequences appropriately usable as this nucleic acid probe include base sequences which are composed of at least 15 bases and encode the amino acid sequences represented by SEQ ID NOs. 9 to 11 in the Sequence Listing or a part of the same. To elevate the detection sensitivity, the nucleic acid probe may be labeled with a radioisotope or a fluorescent substance.

The twenty-second invention relates to the antisense DNA of the above-mentioned gene regulating aureobasidin sensitivity and originating in a mold, while the twenty-third invention relates to the antisense RNA thereof. By introducing this antisense DNA or antisense RNA into cells, the expression of the gene regulating aureobasidin sensitivity can be controlled.

As the antisense DNA to be introduced, use can be made of, for example, the corresponding antisense DNAs of the genes regulating aureobasidin sensitivity represented by SEQ ID NOs. 1, 3 and 12 in the Sequence Listing or a part of the same. SEQ ID NO. 13 in the Sequence Listing shows an example of such an antisense DNA which corresponds to the sequence of the antisense DNA of the gene regulating aureobasidin sensitivity represented by SEQ ID NO. 1 in the Sequence Listing. As the antisense DNA, it is also possible to use fragments obtained by appropriately

cleaving these antisense DNAs or DNAs synthesized on the basis of the sequences of these antisense DNAs.

As the antisense RNA to be introduced, use can be made of, for example, the corresponding antisense RNAs of the genes regulating aureobasidin sensitivity represented by SEQ ID NOs. 1, 3 and 12 in the Sequence Listing or a part of the same. SEQ ID No. 14 in the Sequence Listing shows an example of such an antisense RNA which corresponds to the sequence of the antisense RNA of the gene regulating aureobasidin sensitivity represented by SEQ ID NO. 1 in the Sequence Listing. As the antisense RNA, it is also possible to use fragments obtained by appropriately cleaving these antisense RNAs or RNAs synthesized on the basis of the sequences of these antisense RNAs. For example, use can be made of an RNA prepared by using the corresponding antisense RNA of the gene regulating aureobasidin sensitivity represented by SEQ ID NO. 1 or 3 in the Sequence Listing and treating it with RNA polymerases in an *in vitro* transcription system.

The antisense DNA and antisense RNA can be chemically modified so as to make them hardly degradable *in vivo* and enable them to pass through cell membrane. A substance capable of inactivating mRNA such as a ribozyme may be bound thereto. The antisense DNA and antisense RNA thus prepared are usable in the treatment of various diseases such as mycosis in association with an increase in the content of the mRNA which encodes the gene regulating aureobasidin sensitivity or its functional derivative.

The twenty-fourth invention relates to a recombinant plasmid wherein the gene of the nineteenth invention, which encode a protein regulating aureobasidin sensitivity or its functional derivative and originates in a mold, has been integrated into an appropriate vector. For example, a plasmid wherein an aureobasidin resistant gene has been integrated into an appropriate yeast vector is highly useful as a selective marker gene, since it makes it easy to select a transformant showing the drug resistance against aureobasidin.

Also, a recombinant plasmid can be stably carried *by Escherichia coli,* etc. Examples of the vector usable therefor include pUC118, pWH5, pAU-PS, Traplexll9 and pTB118.

It is also possible to transform a mold by ligating the gene of the nineteenth invention which encodes a protein regulating aureobasidin sensitivity or its functional derivative and originates in a mold to an appropriate vector. When a plasmid such as pDHG25 [Gene, 98, 61-67 (1991)) is

employed as the vector, the DNA introduced into the mold can be maintained therein in the state of the plasmid. When a plasmid such as pSa23 [Agricultural and Biological Chemistry, 51, 2549-2555 (1987)] is employed as a vector, the DNA can be stably maintained in the state of having been integrated into the chromosome of the mold. It is furthermore possible to give a recombinant plasmid for gene expression by reducing the gene of the present invention into the open reading frame (ORF) alone by cleaving it with appropriate restriction enzymes and by ligating it to an appropriate vector. To construct the plasmid for expression, use can be made of a plasmid such as pTV118, etc. (when *Escherichia coli* is employed as the host), pYE2, etc. (when a yeast is employed as the host), pMAMneo, etc. (when mammal cells are employed as a host) or pTAex3, etc. (when a mold is employed as the host) as the vector.

The twenty-fifth invention relates to a transformant obtained by introducing the above-mentioned recombinant plasmid into an appropriate host. As the host, use can be made of *Escherichia coli,* yeasts, molds and mammal cells. *Escherichia coli* JM109 transformed by a plasmid pANAR1 which had the gene anaurl^{s} integrated thereinto was named *Escherichia coli* JM109/pANAR1 and has been deposited at National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology under the accession number FERM BP-5180.

The twenty-sixth invention relates to a process for producing a protein regulating aureobasidin sensitivity or its functional derivative. This process comprises incubating a transformant having the recombinant expression plasmid of the twenty-fourth invention, which contains a gene encoding this protein or its functional derivative, in an appropriate nutritional medium, recovering and purifying the protein thus expressed from the cells or the medium. To express the gene encoding this protein, use is made of *Escherichia coli*, a yeast, a mold or mammal cells as the host.

The twenty-seventh invention relates to a protein regulating aureobasidin sensitivity or its functional derivative. Examples thereof include those encoded by the above-mentioned genes anaurl^{R}, anaurl^{s} and afaurl^{s} and having amino acid sequences represented respectively by SEQ ID NOs. 2, 4 and 5.

As a matter of course, these proteins may have at least one modification selected from

among replacement, insertion and deletion by chemical, physical or genetic engineering techniques. It is also possible to construct an antibody against a protein regulating aureobasidin sensitivity by using the proteins having the amino acid sequences represented by SEQ ID NOs. 2, 4 and 5 or a peptide fragment of a region corresponding to a part of such an amino acid sequence as an antigen.

The twenty-eighth invention relates to a protein capable of imparting aureobasidin resistance wherein at least the amino acid Gly at the position 275 in the gene imparting aureobasidin sensitivity represented by SEQ ID NO. 4 in the Sequence Listing has been replaced by another amino acid. This invention also involves functional derivatives of the same obtained by introducing at least one modification selected from among replacement, insertion and deletion by chemical, physical or genetic engineering techniques thereinto without any injury to the biological activity thereof. The protein of the present invention capable of imparting aureobasidin resistance may be appropriately prepared genetic engineeringly by using DNAs encoding the proteins capable of imparting aureobasidin resistance represented by SEQ ID NOs. 3 and 12 in the Sequence Listing. Its biological activity can be determined by measuring the activity thereof of converting aureobasidin sensitive cells into aureobasidin resistant cells.

The twenty-ninth invention relates to a DNA encoding the protein of the twenty-eighth invention capable of imparting aureobasidin resistance. It also involves DNAs obtained by introducing at least one modification selected from among replacement, insertion and deletion of nucleotide(s) into the above-mentioned DNA. Such a modification may be easily effected by a site-specific mutagenesis. These modified DNAs are employed in order to produce mutated proteins.

The thirtieth invention relates to a method for detecting a gene regulating aureobasidin sensitivity by hybridization with the use of a nucleic acid probe. Examples of the nucleic acid probe usable herein include oligonucleotides which are composed of at least 15 bases and hybridizable selectively with the DNAs represented by SEQ ID NOs. 1, 3 and 12 in the Sequence Listing and fragments thereof. It is appropriate to use therefor base sequences which encode the amino acid sequences represented by SEQ ID NOs. 9 to 11 in the Sequence Listing or a part of the same and consist of at least 15 bases. By using such a nucleic acid probe, DNAs or RNAs extracted from the target organism are subjected to Southern hybridization or Northern hybridization to thereby give the gene of the target organism regulating aureobasidin sensitivity. The nucleic acid probe is also

usable in the confirmation of a tissue wherein the above-mentioned gene can be expressed, or the confirmation of the existence of the gene or mRNA in various vital tissues by *in situ* hybridization.

This nucleic acid probe can be prepared by ligating the above-mentioned gene or its fragment to an appropriate vector, introducing it into a bacterium followed by replication, extracting with phenol, etc. from a disrupted cell solution, cleaving with restriction enzymes capable of recognizing the ligation site with the vector, electrophoresing and excising from the gel. Alternatively, this nucleic acid probe can be prepared by a chemical synthesis with the use of a DNA synthesizer or gene amplification techniques by PCR on the basis of each of the base sequences represented by SEQ ID NOs. 1, 3 and 12 in the Sequence Listing. To elevate the detection sensitivity in use, the nucleic acid probe may be labeled with a radioisotope or a fluorescent substance.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 is a restriction enzyme map of the genes spaur1^R and spaur1^S regulating aureobasidin sensitivity.

Fig. 2 is a restriction enzyme map of scaur1^R and scaur1^S.

Fig. 3 is a restriction enzyme map of scaur2^R and scaur2^S.

Fig. 4 shows the structure of a DNA for disrupting the *Schizo. pombe* spaur1^S gene.

Fig. 5 shows the structure of a DNA for disrupting the *S. cerevisiae* scaur1^S gene.

Fig. 6 shows the results of the detection of the aur1 gene caaur1 carried by *C. albicans* by the PCR method.

Fig. 7 is a restriction enzyme map of the caaur1 gene carried by *C. albicans*.

Fig. 8 is a restriction enzyme map of the caaur2 gene.

Fig. 9 shows the structure of a plasmid YEpSCARW3 for expressing the scaur1 gene.

Fig. 10 shows the results of the northern hybridization of the spaur1 gene of *Schizo. pombe*.

Fig. 11 shows the results of the detection of the scaur1 protein by using an antibody.

Fig. 12 is a restriction enzyme map of pAR25.

Fig. 13 shows a construction of the vector which is a example of the present invention, and integration of the vector into the chromosome.

Fig. 14 shows a pattern of the southern hybridization after electrophoresis of the genomic

DNA digested by a restriction enzyme.

Fig. 15 is a diagram showing the restriction enzyme map of the genomic DNA of a gene anaurl^R regulating aureobasidin sensitivity.

Fig. 16 is a diagram showing the restriction enzyme map of the cDNA of a gene anaurl^S regulating aureobasidin sensitivity.

Fig. 17 is a diagram showing the restriction enzyme map of the cDNA of a gene afaurl^S regulating aureobasidin sensitivity.

Fig. 18 is a diagram showing a comparison among the amino acid sequences of proteins encoded by genes regulating aureobasidin sensitivity.

Fig. 19 is a diagram showing a relation among the genomic DNA, cDNA and protein of a gene anaurl regulating aureobasidin sensitivity.

Fig. 20 is a diagram showing the results of Northern hybridization of genes regulating aureobasidin sensitivity of *A. nidulans* and *A. fumigatus*.

Fig. 21 is a diagram showing the results of Southern hybridization which indicate the detection of genes regulating aureobasidin sensitivity of *A. niger* and *A. oryzae*.

To further illustrate the present invention in greater detail, the following Examples will be given. However it is to be understood that the present invention is not restricted thereto.

EXAMPLES

Example 1: Cloning of a gene regulating aureobasidin sensitivity originating in fission yeast *Schizo. pombe*

1-a) Separation of aureobasidin-resistant mutant of *Schizo. pombe*

About 1 x 10^8 cells of a *Schizo. pombe* haploid cell strain JY745 (mating type h$^-$, genotype ade6-M210, leu1, ura4-D18) exhibiting a sensitivity to aureobasidin at a concentration of 0.08 μg/ml were suspended in 1 ml of a phosphate buffer containing 0.9% NaCl. Then the cells were mutagenized with EMS at a final concentration of 3% at 30°C for 90 minutes. After neutralizing by adding 8 ml of 5% sodium thiosulfate, the cells thus treated were harvested by centrifugation (2500 r.p.m., 5 minutes), washed twice with 6 ml of physiological saline and then suspended in 2 ml of a YEL medium (3% of glucose, 0.5% of yeast extract). The suspension was incubated at 30°C for 5 hours under stirring and then spreaded on a YEA plate (the YEL medium containing

1.5% of agar) containing 5 μg/ml of aureobasidin A. After incubating at 30°C for 3 to 4 days, two or three aureobasidin-resistant colonies were formed per 1 x 10^8 cells. After carrying out the mutagenesis several times, five clone mutants, i.e., THR01, THR04, THR05, THR06 and THR07 were obtained. These mutants were resistant to more than 25 μg/ml of aureobasidin A but the same as the parent strain in the sensitivity to cycloheximide and amphotericin B. Therefore it is estimated that they are not mutants having a multiple drug resistance but ones having a resistance specific to aureobasidin.

1-b) Genetic analysis

Each of the above-mentioned resistant strains THR01, THR04, THR05, THR06 and THR07 was crossed with normal cells of *Schizo. pombe* LH121 strain (mating type h^+, genotype ade6-M216, ura4-D18) differing in mating type. Diploid cells obtained were examined about the resistance to aureobasidin. Similar to the resistant strains, the five diploids formed by crossing the resistant strains with the normal one were resistant to 25 μg/ml of aureobasidin A, thus proving that these resistant mutations were dominant. To perform the tetrad analysis, the above-mentioned diploids were subsequently inoculated on an MEA medium (3% of malt extract, 2.5% of agar) for sporulation and incubated at 25°C for 2 days. Prior to the meiosis, the diploid cells replicated DNA on the MEA medium and then underwent the meiosis to form asci each containing four ascospores of the haploid. These spores were separated with a micromanipulator and allowed to germinate on the YEA plate, followed by the formation of colonies. Then the resistance to aureobasidin of these colonies was examined. Among four spores contained in an ascus, the separation of the sensitivity versus the resistance showed 2:2. This result indicates that the aureobasidin resistant mutation was induced by a mutation in single gene. Further, the complementation test was performed in order to confirm whether the resistant genes of the above-mentioned five mutants were identical with each other or not. For example, a mutant of the mating type h^+, which had been obtained by crossing the mutant THR01 with the LH121 strain in the above tetrad analysis, was crossed with another variant THR04 (mating type h^-) on the MEA plate as described above and, after sporulation, the tetrad analysis was carried out. As a result, all of the colonies formed from four ascospores showed resistance to aureobasidin, which indicates that the mutational genes of THR01 and THR04 are the same with each other. Similarly, the five mutants were examined and it was thus found out that all mutations occurred on the same gene. This gene regulating

aureobasidin sensitivity is named spaur1, the normal gene (sensitive gene) is named spaur1^S and the mutational gene (resistant gene) is named spaur1^R.

1-c) Preparation of genomic library of aureobasidin resistant strain

Genomic DNA was extracted and purified from the aureobasidin resistant strain THR01 by the method of P. Philippsen et al. [Methods in Enzymology, 194, 169 - 175 (1991)]. The purified genomic DNA (8 μg) was partially digested by treating with 5 U of a restriction enzyme HindIII at 37°C for 10 minutes, deproteinized with phenol/chloroform and precipitated with ethanol. The partially digested DNA was electrophoresed on a 0.8% agarose gel and DNA in the region of 3 to 15 kb was extracted. The DNA thus obtained was ligated with a yeast-*E. coli* shuttle vector pAU-PS (2 μg) which had been completely digested with HindIII by using a DNA ligation kit (manufactured by Takara Shuzo Co., Ltd.) and then transformed into *E. coli* HB101. Thus a genomic library of the aureobasidin resistant strain was formed. *E. coli* containing this genomic library was incubated in 50 ml of an LB medium (1% of bacto trypton, 0.5% of bacto yeast extract, 0.5% of sodium chloride) containing 100 μg/ml of ampicillin and 25 μg/ml of tetracycline at 37°C overnight. Then a plasmid was recovered and purified from the *E. coli* cells.

1-d) Expression and cloning of aureobasidin resistant gene spaur1^R

The plasmid originating in the genomic library of the aureobasidin resistant strain as prepared above was transformed into a strain *Schizo. pombe* JY745 by the method of Okazaki et al. [Nucleic Acid Research, 18, 6485 - 6489 (1990)]. The transformed cells were spreaded on a minimum medium SD plate [O.67% of yeast nitrogen base without amino acids (manufactured by Difco), 2% of glucose, 2% of agar] containing 75 μg/ml of adenine sulfate and 50 μg/ml of leucine. After incubating at 30°C for 3 to 4 days, the colonies thus formed were replicated onto an SD plate containing 5 μg/ml of aureobasidin A, 75 μg/ml of adenine sulfate and 50 μg/ml of leucine. It is conceivable that a colony propagated on this plate may have the plasmid containing the aureobasidin resistant gene. This colony was inoculated into 5 ml of a liquid SD medium containing 75 μg/ml of adenine sulfate and 50 μg/ml of leucine. After incubating at 30°C for 2 days, the plasmid was recovered from the propagated cells by the method of I. Hagan et al. [J. Cell Sci., 91, 587 - 595 (1988)]. Namely, the cells were harvested from the culture (5 ml) by centrifugation and then suspended in 1.5 ml of 50 mM citrate/phosphate buffer containing 1.2 M of sorbitol and 2 mg/ml

of Zymolyase. Then the suspension was maintained at 37°C for 60 minutes. The cells were collected by centrifuging at 3,000 r.p.m. for 30 seconds and suspended in 300 μl of a TE [10 mM of Tris-HCl, pH 8, 1 mM of EDTA] solution. After adding 35 μl of 10% SDS, the mixture was maintained at 65°C for 5 minutes. After adding 100 μl of 5 M potassium acetate, the mixture was allowed to stand in ice for 30 minutes. Then it was centrifuged at 10,000 r.p.m. at 4°C for 10 minutes and a plasmid DNA was purified from the supernatant by using EASYTRAP™ (manufactured by Takara Shuzo Co., Ltd.).

This plasmid was transformed into *E. coli* HB101 and a plasmid DNA was prepared from *E. coli* colonies formed on an LB medium containing ampicillin and tetracycline. This plasmid, which contained a DNA of 4.5 kb, was named pAR25. Fig. 12 shows the restriction enzyme map of the DNA of 4.5 kb in pAR25. To specify the gene region, HindIII fragments or SacI fragments of various sizes were subcloned into the pAU-PS vector. These DNAs were transformed into normal JY745 cells by the above-mentioned method of Okazaki et al. and the acquisition of aureobasidin resistance was examined. As a result, it is revealed that a HindIII-SacI 2.4 kb DNA fragment contains the spaur1^R gene. The restriction enzyme map of this DNA segment containing the aureobasidin resistant gene spaur1^R is shown in Fig. 1. This fragment was cloned into a pUC118 vector (named pUARS2R) and then the DNA nucleotide sequence was identified (SEQ ID No. 1 in Sequence Listing). From this nucleotide sequence, it is revealed that the spaur1^R gene code for a protein having an amino acid sequence represented by SEQ ID No. 16 in Sequence Listing.

1-e) Cloning of aureobasidin sensitive gene spaur1^S

By the same method as the one employed in the above c), genomic DNA was extracted and purified from normal cells. After partially digesting with HindIII, a genomic library of the normal cells was constructed. An *E. coli* stock containing this library DNA was spreaded on an LB agar medium containing ampicillin and tetracycline and incubated overnight at 37°C. The colonies thus formed were transferred onto a nylon membrane (Hybond™-N, manufactured by Amersham) and the colony hybridization was performed.

As a probe, the above-mentioned DNA fragment (2.4 kb) obtained by cleaving the spaur1^R gene with HindIII-SacI and labeled with [α-^{23}P] dCTP by using a random primer DNA labeling kit (manufactured by Takara Shuzo Co., Ltd.) was used. As the results of screening of 5 x 10^4

colonies, five clones being hybridizable with the probe were obtained. Plasmids were purified from *E. coli* cells of these five clones. As the result of the cleavage with restriction enzymes, it was found out that all of these clones contained the same DNA fragment of 4.5 kb (named pARN1). The restriction enzyme map of the DNA of 4.5 kb in pARN1 was identical with that of pAR25 shown in Fig. 10. Therefore, a HindIII-SacI 2.4 kb DNA fragment which was a region containing the spaur1^S gene was prepared from pARN1. Then it was cloned into the pAU-PS vector and this plasmid was named pSPAR1.

By using this plasmid pSPAR1, a strain *E. coli* JM109 was transformed and the transformant thus obtained was named and designated as *Escherichia coli* JM109/pSPAR1. It has been deposited at National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology in accordance with the Budapest Treaty under the accession number FERM BP-4485. This DNA fragment containing the aureobasidin sensitive gene spaur1^S had the restriction enzyme map shown in Fig. 1 and the DNA nucleotide sequence thereof was the one represented by SEQ ID No. 17 in Sequence Listing. Based on this nucleotide sequence, it has been revealed that the spaur1^S gene codes for a protein having the amino acid sequence represented by SEQ ID No. 28 in Sequence Listing and, when compared with the resistant gene spaur1^R, the amino acid at the residue 240 has been changed from glycine into cysteine.

Example 2: Cloning of aureobasidin sensitive genes scaur1 and scaur2 originating in budding yeast *S. cerevisiae*

2-a) Separation of aureobasidin resistant mutant of *S. cerevisiae*

A strain *S. cerevisiae* DKD5D (mating type a, genotype leu2-3 112, trp1, his3) having a sensitivity to aureobasidin at a concentration of 0.31 μg/ml was mutagenized with EMS in the same manner as the one employed in the case of *Schizo. pombe*. Then resistant mutants were separated on an agar plate of a complete nutritional medium YPD (1% of yeast extract, 2% of polypeptone, 2% of glucose) containing 5 μg/ml or 1.5 μg/ml of aureobasidin A. After repeating the mutagenesis several times, 34 mutant clones were obtained. These mutants were resistant to more than 25 μg/ml of aureobasidin A and estimated as having not a multiple drug resistance mutation but a aureobasidin-specific resistance mutation.

2-b) Genetic analysis

Similar to the above-mentioned case of *Schizo. pombe*, the genetic analysis using the tetrad analysis and the complementation test was performed. As a result, the genes could be classified into two types. These genes regulating aureobasidin sensitivity were named scaur1 and scaur2, the resistant genes isolated from the resistant mutant were named scaur1^R and scaur2^R, and the sensitive genes isolated from the sensitive wild-type strain were named scaur1^S and scaur2^S, respectively.

The R94A strain had a gene with dominant mutation (scaur1^R). It has been further clarified that the scaur1 gene is located in the neighborhood of the met14 gene of the eleventh chromosome.

2-c) Preparation of genomic library of aureobasidin resistant strain having aureobasidin resistant gene scaur1^R

Genomic DNA was extracted and purified from the aureobasidin resistant strain R94A by the above-mentioned method of P. Philippsen et al. The purified genomic DNA (8 μg) was partially digested by treating with 5 U of a restriction enzyme HindIII at 37°C for 10 minutes, deproteinized with phenol/chloroform and precipitated with ethanol. The partially digested DNA thus obtained was electrophoresed on a 0.8% agarose gel and DNA in the region of 3 to 15 kb was extracted. The DNA thus obtained was ligated with a yeast-*E. coli* shuttle vector pWH5 (2 μg) which had been completely digested with HindIII by using a DNA ligation kit and then transformed into *E. coli* HB101. Thus a genomic library was formed. *E. coli* containing this genomic library was cultured in 50 ml of an LB medium containing ampicillin and tetracycline at 37°C overnight. Then a plasmid was recovered and purified from the *E. coli* cells.

2-d) Expression and cloning of aureobasidin resistant gene scaur1^R

The above-mentioned genomic library of the R94A strain was transformed into *S. cerevisiae* SH3328 (mating type α, genotype ura3-52, his4, thr4, leu2-3 · 112) in accordance with the method of R.H. Schiestl et al. [Current Genetics, 16, 339 - 346 (1989)]. The transformed cells were spread on a minimum medium SD plate [0.67% of yeast nitrogen base without amino acids, 2% of glucose, 2% of agar] containing 25 μg/ml of uracil, 35 μg/ml of histidine and 500 μg/ml of threonine. After incubating at 30°C for 3 to 4 days, the colonies thus formed were replicated onto a YPD agar plate containing 1.5 μg/ml of aureobasidin A. A colony thus formed was inoculated into 5 ml of a liquid YPD medium. After incubating at 30°C for 2 days, a plasmid DNA was recovered from the propagated cells by the above-mentioned method of I. Hagan et al. This plasmid was transformed

into a yeast again and it was confirmed that the obtained transformant had acquired aureobasidin resistance. This plasmid, which contained a DNA of 3.5 kb, was named pWTCR3. Neither the DNA fragment of 2.0 kb nor the DNA fragment of 1.5 kb obtained by cleaving with HindIII exhibited any aureobasidin resistant activity alone. Thus it is confirmed that the gene is contained in the DNA fragment of 3.5 kb. Fig. 2 shows the restriction enzyme map of this DNA fragment of 3.5 kb containing the aureobasidin resistant gene scaur1^R. The HindIII fragments of 1.5 kb and 2 kb were each cloned into pUC118, followed by the determination of the DNA nucleotide sequence (SEQ ID No. 19 in Sequence Listing). From this nucleotide sequence, it has been revealed that the scaur1^R gene codes for a protein having an amino acid sequence represented by SEQ ID No. 20 in Sequence Listing.

2-e) Cloning of aureobasidin sensitive gene scaur1^S corresponding to aureobasidin resistant gene scaur1^R

By the same method as the one employed in the above Example 2-c), genomic DNA was extracted and purified from the parent strain *S. cerevisiae* DKD5D. After partially digesting with HindIII, the DNA was ligated with pWH5 and transformed into *E. coli* HB101. Thus a genomic library of the normal cells was formed. An *E. coli* stock containing this library DNA was spreaded on an LB agar medium containing ampicillin and tetracycline and incubated overnight at 37°C. The colonies thus formed were transferred onto a nylon membrane (Hybond^{TM}-N) and the colony hybridization was carried out. As a probe, the DNA fragment of 3.5 kb obtained in the above Example 2-d) and labeled with [α-^{32}P] dCTP by using a random primer DNA labeling kit (manufactured by Takara Shuzo Co., Ltd.) was used. As the results of screening of 2 x 10^4 colonies, seven clones being hybridizable with the probe were obtained. Plasmids were purified from *E. coli* cells of these clones. As the result of the cleavage with restriction enzymes, one of these clones contained a DNA fragment of 3.5 kb. This DNA fragment had the restriction enzyme map of Fig. 2 and thus judged as containing the scaur1^S gene. The plasmid containing this DNA fragment was named pSCAR1, while *E. coli* HB101 having this plasmid introduced therein was named and designated as *Escherichia coli* HB101/pSCAR1. This strain has been deposited at National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology in accordance with the Budapest Treaty under the accession number FERM BP-4483.

The DNA fragment of 3.5 kb obtained by partially digesting pSCAR1 with HindIII was subcloned into pUC118 and the nucleotide sequence thereof was determined (SEQ ID No. 21 in Sequence Listing). A comparison with the resistant gene indicates that the base at the position 852 has been changed from T into A and, due to this replacement, the amino acid has been converted from phenylalanine into tyrosine (SEQ ID No. 22 in Sequence Listing).

2-f) Preparation of genomic library of aureobasidin resistant strain having aureobasidin resistant gene scaur2^R

A genomic library was prepared from an aureobasidin resistant strain L22-8B by the same method as the one described in Example 2-c). *E. coli* containing this genomic library was cultured in an LB medium (50 ml) containing ampicillin and tetracycline at 37°C overnight. Then plasmids were recovered and purified from the *E. coli* cells.

2-g) Expression and cloning of aureobasidin resistant gene scaur2^R

The above-mentioned plasmids originating in the genomic library of the L22-8B strain were transformed into *S. cerevisiae* SH3328 by the above-mentioned method of R.H. Schiestl. From the transformed strains, an aureobasidin resistant strain was isolated. Then a plasmid DNA containing the scaur2^R gene was recovered from this transformant by the above-mentioned method of I. Hagan et al. This plasmid was transformed into a yeast again and it was confirmed that the transformant had acquired aureobasidin resistance. This plasmid, which contained a DNA of 8.5 kb, was named pSCAR2. Fig. 3 shows the restriction enzyme map of the DNA fragment of 8.5 kb containing this aureobasidin resistant gene scaur2^R. *E. coli* HB101 having this plasmid pSCAR2 introduced therein was named and designated as *Escherichia coli* HB101/pSCAR2. This strain has been deposited at National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology in accordance with the Budapest Treaty under the accession number FERM BP-4484. By using BamHI, EcoRI, HindIII and PstI, DNA fragments of various sizes were prepared and cloned into the pWH5 vector. These plasmids were transformed into *S. cerevisiae* DKD5D in accordance with the above-mentioned method of R.H. Schiestl et al. Then it was examined whether these transformants had acquired aureobasidin resistance or not. As a result, none of the transformants of the DNA fragments was a resistant one. Thus it has been clarified that the DNA fragment of the full length is necessary for the expression of the aureobasidin resistance.

2-h) Isolation of aureobasidin sensitive gene scaur2^S corresponding to aureobasidin resistant gene scaur2^R

An *E. coli* stock containing the genomic library of Example 2-e) prepared from normal cells of *S. cerevisiae* DKD5D was spreaded on an LB agar medium containing ampicillin and tetracycline and incubated at 37°C overnight. The colonies thus formed were transferred onto a nylon membrane (Hybond™-N) and the colony hybridization was performed. As a probe the DNA fragment of 8.5 kb obtained in the above Example 2-g) and labeled with [α-^{32}P] dCTP by using a random primer DNA labeling kit was used. As the results of screening of 2 x 10^4 colonies, several clones being hybridizable with the probe were obtained. Some of these clones contained a DNA fragment of 4.6 kb while others contained a DNA fragment of 3.9 kb. From the restriction enzyme maps of these DNA fragments, it was found out that these DNA fragments were each a part of the scaur2^S gene shown in Fig. 3. These DNA fragments were ligated together to thereby give a scaur2^S fragments shown in Fig. 3. The DNA fragment of 8.5 kb thus obtained was subcloned into pUC118 and then the DNA nucleotide sequence was determined (SEQ ID No. 23 in Sequence Listing). Based on the nucleotide sequence of SEQ ID No. 23 in Sequence Listing, the amino acid sequence represented by SEQ ID No. 24 in Sequence Listing was estimated.

Example 3: Gene disruption test on spaur1^S and scaur1^S genes

3-a) Gene disruption test on spaur1^S gene

In order to examine whether the aureobasidin sensitive gene spaur1^S is necessary in the cell growth by the gene disruption test, the plasmid pUARS2R prepared in Example 1-d) was first cleaved with BalI and EcoT22I. After eliminating a DNA fragment of 240 bp, the residual DNA fragment was blunted by using a DNA blunting kit (manufactured by Takara Shuzo Co., Ltd.). Then this DNA was ligated with a DNA containing ura4$^+$ gene of 1.7 kb, which had been obtained by excising from a pUC8ura4 plasmid [Mol. Gen. Genet., 215, 81 - 86 (1988)] by cleaving with HindIII and blunting, to thereby give a plasmid pUARS2RBT22::ura4-1 and another plasmid pUARS2RBT22::ura4-6 in which the ura4 DNA had been inserted in the opposite direction. Both of these disrupted genes were excised from the vector pUC118 by cleaving with SacI and HindIII and ARS2RBT22::ura4-1 and ARS2RBT22::ura4-6 (Fig. 4), which were spaur1^S DNA fragments containing ura4$^+$, were purified. The purified DNA fragments were transformed into diploid cells

Schizo. pombe C525 (h^{90}/h^{90}, ura4-D18/ura4-D18, leu1/leu1, ade6-M210/ade6-M216) by the above-mentioned method of Okazaki et al. and then a transformant was screened on an SD agar plate containing leucine. In the transformant thus obtained, one of a pair of spaur1^S genes on the chromosome had been replaced by the disrupted gene ARS2RBT22::ura4-1 or ARS2RBT22::ura4-6 introduced thereinto. These cells were allowed to undergo sporulation on a sporulation medium MEA and subjected to the tetrad analysis. As a result, it was found out that two of the four ascospores formed colonies but the residual two spores formed no colony. That is to say, the spores suffering from the replacement of the normal spaur1^S gene by the disrupted gene ARS2RBT22::ura4-1 were not propagated. It has been thus revealed that the spaur1^S gene is essentially required for the growth of the cells.

3-b) Gene disruption test on scaur1^S gene

The plasmid pSCAR1 prepared in Example 2-e) was partially digested with HindIII to thereby give a DNA fragment of 3.5 kb shown in Fig. 2. This DNA fragment was cloned into the HindIII site of pUC119 and the obtained product was named pSCAR3. The obtained pSCAR3 was cleaved with StuI and EcoT22I. After eliminating a DNA fragment of 0.3 kb, the obtained DNA was ligated with a DNA fragment (1.1 kb) of URA3 gene which had been obtained by cleaving a plasmid pYEUra3 (manufactured by Clontech Laboratories, Inc.) with HindIII and EcoRI and blunting. Thus, a plasmid pUSCAR3.ST22::URA3$^+$ and another plasmid pUSCAR3.ST22::URA3A, in which the URA3 gene had been inserted in the opposite direction, were obtained. These disrupted genes were excised in the EcoRI site in the scaur1^S gene and the EcoRI site in the pUC119 vector by cleaving with EcoRI. The scaur1^S DNA fragments containing URA3, SCAR3.ST22::URA3+ and SCAR3.ST22::URA3A (Fig. 5), were purified. The purified DNA fragments were transformed into diploid cells of *S. cerevisiae* AOD1 (mating type a/α, genotype ura3-52/ura3-52, leu2-3 112/leu2-3 112, trp1/TRP1, thr4/THR4, his4/HIS4) by the above-mentioned method of R.H. Schiestl and transformants were screened on an SD agar plate containing leucine. The transformants thus obtained were allowed to undergo sporulation on a sporulation medium SP (1% of potassium acetate, 2% of agar) and subjected to the tetrad analysis. As a result, it was found out that two of the four ascospores underwent germination and formed colonies but the residual two spores did not undergo colony formation. That is to say, the spores

suffering from the replacement of the scaur1^S gene by the disrupted gene were not propagated. It has been thus revealed that the scaur1^S gene is essentially required for the growth of the cells.

Example 4: Examination on the expression of aureobasidin sensitive gene spaur1 by northern hybridization

From a normal strain or a resistant strain of *Schizo. pombe*, the whole RNAs were extracted and purified by the method of R. Jensen et al. [Proc. Natl. Acad. Sci. USA, 80, 3035 - 3039 (1983)]. Further, poly(A)$^+$RNA was purified by using Oligotex™-dT30 (manufactured by Takara Shuzo Co., Ltd.). The purified poly(A)$^+$RNA (2.5 μg) was separated by the electrophoresis on a 1.2% agarose gel containing formaldehyde and transferred onto a nylon membrane (Hybond™-N). After immobilizing, the hybridization was performed with the use of a HindIII-SacI fragment (2 kb) of the spaur1^R gene labeled with [α-^{32}P]dCTP as a probe. As a result, both of the normal cells and the resistant cells showed a band of the same amount of about 2 kb. In both cases, this amount underwent no change in the logarithmic growth phase and the stationary phase (Fig. 10). Fig. 10 is an autoradiogram showing the results of the northern hybridization wherein mRNAs obtained from cells of a sensitive strain of *Schizo. pombe* in the logarithmic growth phase (lane 1), cells of a resistant strain in the logarithmic growth phase (lane 2), cells of the sensitive strain in the stationary phase (lane 3) and cells of the resistant strain in the stationary phase (lane 4) are electrophoresed on a 1.2% agarose gel containing formaldehyde.

Example 5: Determination of the activity of scaur1^S gene

5-a) Construction of plasmid YEpSCARW3 (Fig. 9) and YEpSCARW1

The plasmid pSCAR1 prepared in Example 2-e) was cleaved with HindIII and a fragment of 2 kb containing the whole ORF was excised. This fragment was inserted into the HindIII site of a expression-plasmid YEp52 having a promoter Gal10, the expression of which was induced by galactose in a medium. The plasmid having the scaur1^S gene which had been inserted in such a direction as to be normally transcribed by the promoter Gal10 was named YEpSCARW3. Fig. 9 shows the structure of this plasmid. Further, the plasmid having the scaur1^S gene inserted in the opposite direction was named YEpSCARW1.

5-b) Transformation by plasmids YEpSCARW3 and YEpSCARW1

By using 5 μg portions of the plasmids YEpSCARW3 and YEpSCARW1, the diploid *S.*

cerevisiae cells with the disrupted scaur1^S gene prepared in Example 3-b) were transformed. Then transformants were screened on an SD agar plate. These transformants were allowed to undergo sporulation on an SP medium and then subjected to the tetrad analysis. When the expression of the scaur1^S gene was induced by using a YPGal medium (1% of yeast extract, 2% of polypeptone, 2% of galactose), the ascospores formed from the diploid cells transformed by YEpSCARW3 all underwent germination while two of the four ascospores formed from the diploid cells transformed by YEpSCARW1 underwent germination but not the remaining two. It is thus conceivable that the cells with the disrupted scaur1^S gene have reverted to the normal state by introducing YEpSCARW3 containing the scaur1^S gene into these cells. Accordingly, the use of these cells with the disrupted scaur1^S gene as a host makes it possible to determine the activity of normal aur1-analogous genes carried by other organisms.

Example 6: Confirmation and cloning of aur1 and aur2 genes (caaur1, caaur2) carried by *C. albicans*

6-a) Detection of aur1 gene by the PCR method

Poly(A)$^+$RNA was extracted and purified from an aureobasidin sensitive strain *C. albicans* TIMM0136 by the same method as the one employed in Example 4. By using the poly(A)$^+$RNA (5 μg) as a template, a double-stranded cDNA was synthesized on a cDNA synthesizing system Plus (manufactured by Amersham) with the use of an oligo(dT) primer. Mixed primers for PCR corresponding to amino acid sequence regions being common to the amino acid sequences of *S. cerevisiae* and *Schizo. pombe* were synthesized on a DNA synthesizer and purified. That is to say, a primer of SEQ ID No. 25 in Sequence Listing corresponding to the region of amino acids at the 184- to 192-positions of SEQ ID No. 18 in Sequence Listing of *Schizo. pombe* (from the 184- to 192-positions of SEQ ID No. 22 in Sequence Listing of *S. cerevisiae*) and another primer of SEQ ID No. 26 in Sequence Listing corresponding to the region of amino acids from the 289- to 298-positions of *Schizo. pombe* (from the 289- to 298-positions of SEQ ID No. 22 in Sequence Listing of *S. cerevisiae*) were employed.

PCR was performed by using these primers and the above-mentioned cDNA as a template by repeating a cycle comprising treatment at 94°C for 30 seconds, one at 48°C for 1 minute and one at 72°C for 2 minutes 25 times. As a result, a DNA (about 350 bp) being almost the same as

S. cerevisiae and *Schizo. pombe* in length was amplified (Fig. 6). Fig. 6 shows a pattern obtained by carrying out PCR with the use of cDNA of *C. albicans* (lane 1), cDNA of *S. cerevisiae* (lane 2) and cDNA of *Schizo. pombe* (lane 3) as a template, electrophoresing each PCR product on an agarose gel and staining with ethidium bromide.

6-b) Cloning of aur1 gene (caaur1) of *C. albicans*

(i) Genomic DNA was extracted and purified from a strain *C. albicans* TIMM0136 by the same method as the one described in Example 1-c). After partially digesting with HindIII, the DNA fragment was ligated with a Traplexll9 vector which had been completely digested with HindIII and transformed into *E. coli* HB101. Thus a genomic library of *C. albicans* was prepared. From this library, a DNA fragment of 4.5 kb containing the aur1 gene of *C. albicans* was cloned by using the DNA fragment of *C. albicans* obtained by the PCR described in Example 6-a), which had been labeled with [α-^{32}P]dCTP by using a random primer DNA labeling kit (manufactured by Takara Shuzo Co., Ltd.), as a probe. This DNA fragment had a restriction enzyme map shown in Fig. 7 and the DNA nucleotide sequence thereof is represented by SEQ ID No. 27 in Sequence Listing. Based on this nucleotide sequence, it was estimated that the caaur1 gene coded for a protein having the amino acid sequence represented by SEQ ID No. 28 in Sequence Listing. When compared with the scaur1^{S} protein, a homology of as high as 53% was observed. A Traplexll9 vector having this caaur1 gene integrated therein was named pCAAR1, while *E. coli* HB101 transformed by this plasmid was named and designated as *Escherichia coli* HB101/pCAAR1. This strain has been deposited at National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology in accordance with the Budapest Treaty under the accession number FERM BP-4482.

Next, pCAAR1 was treated with HindIII to thereby give caaur1 of 4.5 kb. Further, it was integrated into pTV118 which had been completely digested with HindIII to thereby prepare a plasmid for expressing caaur1. This plasmid was named pTCAAR1. (ii) Genomic DNA was extracted and parified from a strain *C. albicans* TIMM1768 [The Journal of Antibiotics, 46, 1414-1420(1993)] by the same method as the one described in Example 1-c). After partially digesting with HindIII, the DNA fragment was ligated with a pUC118 vector which had been completely digested with HindIII and transformed into *E. coli* HB101. Thus a genomic library of

C. albicans TIMM1768 was prepared. From this library, a DNA fragment of 4.5 kb containing the aur1 gene of *C. albicans* TIMM1768 was cloned by the colony hybridization with the same probe as that described in Example 6-b)-(i). This DNA fragment had the same restriction enzyme map as that shown in Fig. 7. Next, a part of the DNA sequence containing a ORF in this DNA fragment was determined. The DNA nucleotide sequence thereof is represented by SEQ ID No. 35 in Sequence Listing. Based on this nucleotide sequence, it was estimated that this gene coded for a protein having the amino acid sequence represented by SEQ ID No. 36 in Sequence Listing. When the amino acid sequence of the caaur1 protein *C. albicans* TIMM1768 was compared with that of the caaur1 protein of *C. albicans* TIMM0136, the amino acid sequences of the 1- to 381-positions and the 383- to 423-positions and the 425- to 471-positions of caaur1 protein (SEQ ID No. 28 in Sequence Listing) in *C. albicans* TIMM0136 were identical with the amino acid sequences of the 2- to 382-positions and the 384- to 424-positions and the 426- to 472-positions, respectively, of caaur1 protein (SEQ ID No. 36 in Sequence Listing) in *C. albicans* TIMM1768.

However, serines at the 382- and 424-positions of SEQ ID No. 28 in Sequence Listing were replaced with prolines at the 383- and 425-positions of SEQ ID No. 36 in Sequence Listing.

6-c) Cloning of aur2 gene (caaur2) of *C. albicans*

Genomic DNA of a strain C. albicans TIMM0136 was digested with BamHI and ligated with a pTV118 vector which had been completely digested with BamHI. Then it was transformed into *E. coli* HB101 to thereby prepare a genomic library of *C. albicans*. On the other hand, the DNA fragment containing the scaur2^S gene obtained in Example 2-h) was cleaved with HindIII and PstI to thereby give a DNA fragment of 1.2 kb. This DNA fragment was labeled with [α-^{32}P]dCTP by using a random primer DNA labeling kit. By using this labeled DNA fragment as a probe, the above-mentioned *C. albicans* genomic library was screened by the colony hybridization. Thus a plasmid containing a DNA fragment of 8.3 kb was obtained. A part of the DNA sequence upstream of the BamHI site of this DNA fragment was determined (SEQ ID No. 29 in Sequence Listing). Based on this sequence, an amino acid sequence represented by SEQ ID No. 30 in Sequence Listing was estimated. It corresponded to the amino acid sequence of the 1230- to 1309-positions of the amino acid sequence of the scaur2 gene (SEQ ID No. 24), having a homology of as high as 77%. Since this DNA fragment lacked a part of the C-end, the genomic library prepared in Example 6-b)

was further screened by using this DNA fragment as a probe. Thus a DNA fragment of 6.5 kb having the C-terminal part was obtained. Fig. 8 shows the restriction enzyme map of the DNA region containing the caaur2 gene thus clarified.

A pTV118 vector having the above-mentioned caaur2 gene of 8.3 kb integrated therein was named pCAAR2N, while *E. coli* HB101 transformed by this plasmid was named and designated as *Escherichia coli* HB101/pCAAR2N. This strain has been deposited at National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology in accordance with the Budapest Treaty under the accession number FERM BP-4481.

Example 7: Preparation of antibody against protein coded for by scaur1^S gene and staining of *S. cerevisiae* cells and detection of said protein by using this antibody.

7-a) Preparation of antibody

SCAR1-1 (SEQ ID No. 33 in Sequence Listing) comprising a peptide corresponding to the amino acids at the residue 103 to 113 in the amino acid sequence of SEQ ID No. 22 in Sequence Listing having cysteine added to the N-end thereof and SCAR1-2 (SEQ ID No. 34 in Sequence Listing) comprising a peptide corresponding to the amino acids at the residue 331 to 348 in the amino acid sequence of SEQ ID No. 22 having cysteine added to the N-end thereof were synthesized by the Fmoc solid phase synthesis method and purified by reverse phase HPLC. Thus 10 mg portions of these peptides were obtained. To the N-terminal cysteine of each of these synthetic peptides, KLH was bound as a carrier protein. By using this binding product as an antigen, a rabbit was immunized and an antiserum was obtained. This antiserum was further purified on an affinity column prepared by binding the synthetic peptide employed as the antigen to an agarose gel. Thus a polyclonal antibody being specific for the synthetic peptide was prepared.

7-b) Staining of *S. cerevisiae* cells with antibody

A strain *S. cervisiae* ATCC 9763 was cultured in a YNBG medium [0.67% of yeast nitrogen base (manufactured by Difco), 2% of glucose] to thereby give a suspension of a concentration of 3×10^7 cells/ml. To 1 ml of this cell suspension were added 0.11 ml of a 1 M phosphate buffer (pH 6.5) and 0.17 ml of 37% formaldehyde. After slowly stirring at room temperature for 1 hour, the cells were harvested by centrifugation and then suspended in 20 ml of an SS buffer (1 M of sorbitol, 0.2 % of β-mercaptoethanol, 0.1 M phosphate buffer, pH 7.5) containing 20 μg/ml of Zymolyase

20T. After treating at 30°C for 1 hour, the cells were harvested, washed with the SS buffer, suspended in 1 ml of the SS buffer containing 0.1% of Triton X-100 and then allowed to stand for 10 minutes. This cell suspension was placed on a slide glass which had been coated with poly(L-lysine)and allowed to stand for 10 minutes. Next, a PBS solution containing 1% of albumin (BSA) was dropped thereinto. After allowing to stand at room temperature for 15 minutes, the excessive liquid was removed and then a PBS solution containing BSA containing 0.02 mg/ml of the antiSCAR1-1 antibody was dropped thereinto. After allowing to stand at room temperature for 60 minutes and washing with PBS containing BSA three times, antirabbit IgG antibody labeled with FITC (antibody concentration 0.02 mg/ml) was layered over and allowed to stand at room temperature for 1 hour. After washing with a PBS solution containing BSA, a small amount of a mounting solution, which was a solution prepared by dissolving 0.1 g of p-phenylenediamine in 10 ml of CBS (150 mM of NaCl, 50 mM of CHES, pH 9.5), adjusting the pH value to 9.0 with 10 N NaOH and further adding 90 ml of glycerol, was layered over. Then a cover glass was placed thereon to thereby give a specimen. This specimen was observed under a fluorescence microscope to thereby examine the intracellular distribution of the scaur1 protein. As a result,it was found out that this protein was distributed all over the cells.

7-c) Detection of protein coded for by scaur1 gene by using antibody

The plasmid YEpSCARW3 prepared in Example 5-a) was introduced into a normal haploid *S. cerevisiae* SH3328 to thereby give a transformant. This transformant was cultured in a YPGal medium or a YPD medium and the cells were harvested by centrifugation. The cells thus obtained were suspended in a buffer (1% of Triton X-100, 1% of SDS, 20 mM of Tris-HCl, pH 7.9, 10 mM of EDTA, 1 mM of DTT, 1 mM of PMSF). Further, glass beads were added thereto to disrupt the cells by vigorous vortex. Then an SDS loading solution was added thereto and the protein was denatured by treating at 95°C for 5 minutes. After centrifuging, a part of the obtained supernatant was subjected to SDS-PAGE and the protein thus separated was transfered onto an Immobilon membrane (manufactured by MILLIPORE). This Immobilon membrane was treated with Block Ace (manufactured by Dainippon Pharmaceutical Co., Ltd.). Then the antiSCAR1-2 antibody prepared in 7-a) was reacted therewith as a primary antibody. After washing, antirabbit IgG antibody labeled with peroxidase was reacted therewith as a secondary antibody and the mixture

was thoroughly washed. Next, it was color-developed with diaminobenzidine and a band of the scaur1 protein was detected. Fig. 11 shows the results.

Fig. 11 shows the results of the detection of the protein prepared from the cells incubated in the YPD medium (lane 1) and the protein prepared from the cells incubated in the YPGal medium (lane 2), each subjected to SDS-PAGE, by using the antiSCAR1-2 antibody. The cells incubated in the YPGal medium, of which scaur1 gene had been induced, showed a specific band.

Example 8: Construction of chromosome integration vector containing aureobasidin resistant gene

8-a) Construction of replication vector containing scaur1^R

A plasmid pSCAR1 was prepared from *Escherichia coli* HB101/pSCAR1 (FERM BP-4483) which carried a plasmid pSCAR1 containing scaur1^S. Then the obtained plasmid was partially cleaved with HindIII and thus a DNA of 3.5 kb containing scaur1^S was separated therefrom. This DNA (3.5 kb) was ligated to a vector pUC118 cleaved with HindIII to thereby prepare a plasmid pUscaur1^S. This plasmid pUscaur1^S was transformed into *Escherichia coli* CJ236 to thereby prepare ssDNA.

Next, a site-specific DNA mutation was introduced by using Mutan-K kit (manufactured by Takara Shuzo Co., Ltd.) with the use of a synthetic oligonucleotide for introducing mutation represented by SEQ ID No. 37 in the Sequence Listing, which had been synthesized and purified, and the above-mentioned ssDNA. That is to say, the use of the oligonucleotide represented by SEQ ID No. 37 in the Sequence Listing made it possible to obtain scaur1^R wherein the codon TTT of the 158th amino acid residue Phe in the ORF of the gene scaur1^S had been replaced by the codon TAT of Tyr. This plasmid having a DNA coding for Aur1^{R}p (F158Y) was designated as pUscaur1R.

8-b) Amplification of scaur1^R by PCR method

By using the plasmid pUscaur1^R which carried a HindIII fragment of 3.5 kb containing scaur1^R, scaur1^R (about 1.9 kb) was amplified by the PCR method. Regarding primers employed herein, XhoI and KpnI sites had been designed in primers in order to clone the amplified scaur1^R into the plasmid vector pYES2 (manufactured by Invitrogen corporation) and thus primers represented by SEQ ID Nos. 38 and 39 in the Sequence Listing were synthesized.

The reaction was effected in the following manner. 100 μl of a PCR solution containing 28

μl of a PCR buffer [capable of giving final concentrations of 10 mM of Tris-HCl (pH 8.3), 50 mM of KCl, 1.5 mM of $MgCl_2$, 0.1 mM of dATP, 0.1 mM of dCTP, 0.1 mM of dTTP and 0.1 mM of dGTP], 1 μl of 2.5 U of Ampli Taq DNA polymerase (Manufactured by Perkin-Elmer), 0.5 μl portions of 20 pmol of the primers represented by SEQ ID Nos. 38 and 39 in the Sequence Listing, 1 μl of the plasmid and 69 μl of H_2O was maintained at an initial temperature of 94°C for 1 minute, and then heated successively at 94°C for 1 minute, at 50°C for 2 minutes and at 72°C for 3 minutes. This heating cycle was repeated 35 times. Next, the reaction mixture was maintained at 72°C for 10 minutes to thereby effect the amplification by PCR. Then the PCR amplification product was cleaved with KpnI and XhoI and electrophoresed on an agarose gel and the target DNA fragment of about 1.9 kb was recovered from the gel and purified by using Suprec™-01 (manufactured by Takara Shuzo Co., Ltd.).

8-c) DNA ligation and transformation

About 0.3 μg of the DNA fragment (about 1.9 kb) purified in the above step was ligated to about 0.1 μg of pYES2, which had been digested with XhoI and KpnI, by using a DNA ligation kit (manufactured by Takara Shuzo Co., Ltd.).

Next, 7 μl of the above-mentioned ligation mixture was added to 200 μl of competent cells of *Escherichia coli* HB101. These cells were allowed to stand on ice for 30 minutes, at 42°C for 1 minute and then on ice again for 1 minute. Then 800 μl of an SOC medium [Sambrook et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press (1989)] was added thereto. After incubating at 37°C for 1 hour, these *Escherichia coli* cells were spread onto an L-broth agar medium containing 50 μg/ml of ampicillin and incubated at 37°C overnight. Thus a transformant was obtained.

This transformant was incubated in 5 ml of an L-broth medium containing 50 μg/ml of ampicillin at 37°C overnight. From this culture, a plasmid DNA was prepared in accordance with the alkali method (Molecular Cloning, cited above). The plasmid thus obtained was named pYES2aur1.

8-d) Construction of chromosome integration vector

About 0.4 μg of the above-mentioned plasmid pYES2aur1 was digested with XbaI and KpnI and electrophoresed on an agarose gel. Then a DNA fragment of about 1.9 kb containing scaur1^R

was recovered from the gel and purified by using Suprec-O1.

Similarly, about 0.4 μg of the plasmid vector pUC19 was digested with SspI and PvuII and electrophoresed on an agarose gel. Then a DNA fragment (about 1.8 kb) containing an ampicillin resistant gene and ColE1 origin was recovered from the gel and purified. About 0.1 μg portions of these DNA fragments thus purified were blunt-ended with the use of a DNA blunting kit (manufactured by Takara Shuzo Co., Ltd.). Further, about 0.1 μg portions of these blunted DNA fragments were subjected to a ligation reaction. The ligation reaction was effected by using a DNA ligation kit (manufactured by Takara Shuzo Co.,Ltd.).

Subsequently, the plasmid was integrated into *Escherichia coli* JM109. After incubating, a transformant and a plasmid DNA were prepared. The plasmid thus obtained was named plasmid pAUR1. Next, this plasmid was cleaved with StuI to thereby prepare a chromosome integration vector.

Example 9: Construction of chromosome integration vector containing aureobasidin resistant gene

9-a) Amplification of DNA fragment of scaur1^R having mutation introduced therein by PCR

In order to replace the 240th amino acid residue Ala of Aur1^{R}p (F158Y) by Cys, a primer, wherein the codon GCT of Ala had been changed into the codon TGT of Cys, represented by SEQ ID No. 48 in the Sequence Listing was synthesized and purified. By using the plasmid pUscaur1R described in Example 8-a), which carried a HindIII fragment (3.5 kb) containing scaur1^R at the HindIII site of pUC119, as a template, was amplified a DNA fragment (about 1.4 kb) which contained a sequence of about 500 bp coding for the amino acid sequence on the C-terminal side of Aur1^{R}p (F158Y, A240C), wherein GCT had been changed into TGT, by the PCR method with the use of the primer represented by SEQ ID No. 48 in the Sequence Listing and a primer M13M4. The PCR was effected in the following manner. To 28 μl of a PCR buffer [capable of giving final concentrations of 10 mM of Tris-HCl (pH 8.3), 50 mM of KCl, 1.5 mM of $MgCl_2$, O.1 mM of dATP, O.1 mM of dCTP, O.1 mM of dTTP and O.1 mM of dGTP] were added 2.5 U of Ampli Taq DNA polymerase, 100 pmol portions of the primer represented by SEQ ID No. 48 in the Sequence Listing and the primer M13M4, 1 ng of pUscaur1R and distilled water to thereby give 100 μl of a PCR solution. This reaction mixture was then heated successively at 94°C for 1 minute, at 55°C

for 1.5 minutes and at 72°C for 1.5 minutes. This cycle was repeated 30 times. Next, the PCR product was cleaved with SalI and SnaI and electrophoresed on an agarose gel. The target DNA fragment of about 1.3 kb was recovered from the gel and purified.

9-b) Construction of plasmid containing DNA coding for Aur1^{R}p (F158Y, A240C)

pUscaur1R was cleaved with SalI and SnaI and electrophoresed on an agarose gel. The target DNA fragment of 5.3 kb was recovered and purified. To this DNA fragment was ligated the DNA fragment of 1.3 kb obtained in Example 9-a). The obtained plasmid, which had a DNA (scaur1^R-C) coding for Aur1^{R}p (F158Y, A240C), was named pUscaur1^R-C. To effect transformation by integrating it into the chromosome of sake yeast, pUscaur1^R-C was linearized by cleaving with StuI prior to use. As a control, pUscaur1R was also linearized with StuI.

9-c) Construction of plasmid containing DNA coding for Aur1^{R}p (A240C)

pUscaur1^S was cleaved with SalI and SnaI and electrophoresed on an agarose gel. The target DNA fragment of 5.3 kb was recovered and purified. This DNA fragment was ligated the DNA fragment of 1.3 kb obtained in Example 9-a). The obtained plasmid, which had a DNA coding for Aur1^{R}p (A240C) wherein the 240th residue Ala of Aur1^{S}p had been replaced by Cys, was named pUscaur1A240C. To effect transformation by integrating it into the chromosome of sake yeast, pUscaurA240C was linearized by cleaving with StuI prior to use.

Example 10: Transformation by using sake yeast as host

10-a) About 10 μg of the linearized vector of the plasmid pAUR1 described in Example 8 was introduced into Sake yeast Kyokai k-701 by the lithium acetate method [Journal of Bacteriology, 153, 163 (1983)].

Namely, to Sake yeast Kyokai K-701, which had been suspended in a 0.1 M lithium acetate solution (about 1.3 x 10^8 cells/100 μl of 0.1 M lithium acetate), was added 10 μg of the vector which had been prepared through the linearization of scaur1^R by cleaving with StuI at one position. After treating at 30°C for 30 minutes and then at 42°C for 15 minutes, the cells were harvested by centrifugation and pre-incubated in 5 ml of a YPD liquid medium. After pre-incubating in a YPD liquid medium containing 0.4 μg/ml of aureobasidin A, transformants were obtained on a YPD agar medium containing 0.8 μg/ml of aureobasidin A. This transformant was named Sake yeast Kyokai K-701/pAUR1.

This transformant was subcultured over three generations in the absence of aureobasidin A and then the sensitivity to aureobasidin was assayed. As a result, it showed eight times as much aureobasidin resistance (MIC 1.56 μg/ml) as that of the parent strain (i.e., Sake yeast Kyokai K-701), which indicated that the aureobasidin resistance was sustained. Thus it has been confirmed that the aureobasidin resistance introduced on the host chromosome is usable as a selective marker.

10-b) To compare the activities of pUscaur1^R-C prepared in Example 9-b), pUscaur1A240C prepared in Example 9-c) and pUscaur1R prepared in Example 8-a), 5 μg of the plasmid, which had been linearized with StuI, was introduced into Sake yeast Kyokai K-701 by the lithium acetate method. Namely, to sake yeast, which had been suspended in a 0.1 M lithium acetate solution (pH 7.5) and made competent, were added 5 μg of the plasmid, which had been linearized by cleaving with StuI at one position, and 850 μl of 40% polyethylene glycol/O.1 M lithium acetate. After treating at 30°C for 30 minutes and then maintaining at 42°C for 15 minutes, the cells were harvested, pre-incubated in 5 ml of a YPD liquid medium for 1 hour or overnight and then smeared on a YPD agar medium containing aureobasidin A at various concentration. After incubating at 30°C for 3 to 4 days, transformants having a resistance to aureobasidin A were obtained. As Table 4 shows, the transformant prepared by using the StuI-linearized pUscaur1^R-C could grow even in the medium containing 5 μg/ml of aureobasidin A. These transformants sustained an aureobasidin A resistance at least 10 times higher than that of the parent strain even after being subcultured over several generations. The transformant obtained by using the linearized pUscaur1^R-C showed an MIC to aureobasidin A of 20 μg/ml or above. Thus it has been confirmed that the Aur1^{R}p (F158Y, A240C) is usable as an effective selective marker for sake yeast. As Table 4 shows, the StuI-linearized pUscaur1A240C exceeded the StuI-linearized pUscaur1R in the activity of imparting resistance. That is to say, the mutation at the 240th residue Ala resulted in the expression of the activity of imparting a stronger resistance.

Table 4

Plasmid	Pre-incubation time	No. of transofrmants/μg DNA Aureobasidin A concn. (μg/ml) 0.5	1.0	5.0
StuI-linearized	1 hour	0	0	0
pUscaur1R	overnight	5	4	0
StuI-linearized	1 hour	170	73	0
pUscaur1R-C	overnight	2368	2024	64
StuI-linearized	1 hour	18	1	0
pUscaur1A240C	overnight	160	152	0
no plasmid	1 hour	0	0	0
(control)	overnight	0	0	0

Example 11: Transformation by chromosome integration vector having aureobasidin resistant gene and AARE gene

11-a) Construction of plasmid containing AARE gene

0.5 μg of the plasmid pAUR1 was cleaved with SphI and electrophoresed on an agarose gel. Then the linearized plasmid pAUR1 was recovered from the gel and purified.

Next, a plasmid pYHA201 was prepared from a yeast BJ2168, which carried a plasmid pYHA201 containing the AARE gene described in Japanese Patent Laid-Open No. 254680/1991 (i.e., *Saccharomyces cerevisiae* BJ2168/pYHA201; FERM P-11570). 0.5 μg of this plasmid pYHA201 was digested with SphI and the DNA fragments (about 3.2 kb) were isolated and purified.

This DNA fragment of about 3.2 kb contained the AARE gene bound to the downstream of the ADHI promotor.

By using each 0.2 μg portions of both the purified SphI-cleaved DNA fragments, a ligation

reaction was effected by using a DNA ligation kit (manufactured by Takara Shuzo Co., Ltd.). Subsequently, the plasmid was integrated into *Escherichia coli* JM109 and incubated to thereby give a transformant and a plasmid DNA. The plasmid thus obtained was named plasmid pAUR1aare, while the transformant thus obtained was named and indicated as *Escherichia coli* JM109/pAUR1aare and has been deposited at National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology under the accession number FERM P-14366.

11-b) Expression of AARE in sake yeast

By using about 10 μg of the above-mentioned plasmid pAUR1aare, the plasmid pAUR1aare linearized was transformed into Sake yeast Kyokai K-701 in the same manner as the one described in Example 10. Then an aureobasidin resistant transformant was obtained on a YPD agar medium containing 0.4 μg/ml of aureobasidin A.

The transformant thus obtained was named and indicated as *Saccharomyces cerevisiae* K701/pAUR1aare and has been deposited at National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology under the accession number FERM P-14379.

Next, this transformant was inoculated into 5 ml of a minimal medium [0.67% of Bacto Yeast Nitrogen Base w/o Amino Acid (manufactured by Difco), 2% of glucose] and incubated at 30°C for two days and then the cells were harvested by centrifugation.

After discarding the supernatant, the cells were washed with 2 ml of water and harvested again by centrifugation.

Subsequently, the cells were suspended in 700 μl of a 0.2 M sodium phosphate buffer Solution (pH 7.2).

To this suspension were added 400 μl of glass beads (0.40 to 0.60 mm in diameter) and the cells were disrupted by vigorously stirring under ice-cooling.

After centrifuging, the supernatant was recovered and regarded as a extract.

Also, extracts of Sake yeast Kyokai K-701 and Sake yeast Kyokai K-701/pAUR1 obtained in Example 10 were prepared in the same manner.

The acylamino acid releasing enzyme activity of each of the extracts thus obtained was

measured by the following method. 0.89 ml of a 0.5% dimethylformamide-0.2 M sodium phosphate buffer solution (pH 7.2) containing 0.020 mM of an amide prepared from N-acetyl-L-methionine and 7-amino-4-methylcoumarin (AMC) was preheated at 37°C for about 5 minutes. Then 100 µl of the above-mentioned extract was added thereto and the resulting mixture was incubated at 37°C for 15 minutes. After the completion of the reaction, 10 µl of 10% SDS was added to thereby cease the reaction and the intensity of fluorescence was measured with a fluorophotometer. Namely, the excitation wavelength and the measurement wavelength were set respectively to 380 nm and 440 nm. The amount of the liberated AMC was determined by preparing a standard curve with the use of AMC samples of known concentrations and comparing the obtained data therewith.

100 µl of the extract of *S. cerevisiae* K701/pAUR1aare had an activity of liberating about 25 pmol of AMC in 15 minutes in the above reaction system.

On the other hand, the extract of Sake yeast Kyokai K-701 having no plasmid and the extract of Sake yeast Kyokai K-701/pAUR1 obtained in Example 10 showed each no AARE activity.

Further, an analysis was effected by the southern hybridization with the use of the aureobasidin resistant gene as a probe. As a probe in the hybridization, use was made of an scaur1^R fragment (about 1.6 kb) which had been amplified by the PCR method with the use of the plasmid pAUR1 as a template and the primers represented by SEQ ID Nos. 40 and 41 in the Sequence Listing. 100 ng of the fragment thus obtained was labeled with [^{32}P]dCTP by using a BcaBEST™ labeling kit. The genomic DNAs of Sake yeast Kyokai K-701 and *Saccharomyces cerevisiae* K701/pAUR1aare were cleaved with various restriction enzymes (HPaI having no cleavage site on pAUR1aare, BamHI having two cleavage sites on pAUR1aare), electrophoresed on an agarose gel and transferred onto the hybridization filter.

In Fig. 14, the lanes 1 and 2 show the results obtained by cleaving the genomic DNA of Sake yeast Kyokai K-701 with HpaI (lane 1) or BamHI (lane 3) and subjected to southern hybridization, while the lanes 2 and 4 show the results obtained by cleaving the genomic DNA of *Saccharomyces cerevisiae* K701/pAUR1aare with HpaI (lane 2) or BamHI (lane 4) and subjected to southern hybridization. Since HpaI had no cleaving site on the plasmid pAUR1aare but cleaved exclusively the genomic DNA, it was proved from the lanes 1 and 2 that the aureobasidin resistant gene had

been integrated into one of a pair of chromosomes.

It was confirmed from the lanes 3 and 4 that the aureobasidin resistant gene had been homologously integrated into the aureobasidin sensitive gene on the chromosome of the sake yeast.

These results indicate that genes of the sake yeast were not disrupted by the random integration of the aureobasidin resistant gene.

As described above, by using the chromosome integration vector of the present invention, the resistance could be imparted to an aureobasidin sensitive fungi and, furthermore, a foreign gene could be expressed.

Example 12: Construction of recombinant plasmid containing aureobasidin resistant gene

12-a) Construction of pYC vector carrying DNA coding for Aur1^{R}p (F158Y, A240C)

By employing pUscaur1^R-C as a template, PCR was effected with the use of primers represented by SEQ ID Nos. 49 and 59 in the Sequence Listing. The PCR product (about 2.2 kb), which contained the DNA coding for the amplified Aur1^{R}p (F158Y, A240C), was cleaved with XhoI and KpnI and blunt-ended with the use of a blunting kit (manufactured by Takara Shuzo Co., Ltd.).

A pYC vector pYEUra3 (manufactured by Clontech Laboratories, Inc.) was cleaved with EcoRI and BamHI and blunt-ended with the use of a blunting kit. Then it was ligated to the blunt-ended PCR product (about 2.2 kb) described above. The plasmid thus obtained was named pYCscaur1^R-C. By employing pUscaur1R as a template, PCR was effected in the same manner with the use of primers represented by SEQ ID Nos. 49 and 50 in the Sequence Listing. The PCR product (about 2.2 kb), which contained the DNA coding for the amplified Aur1^{R}p (F158Y), was cleaved with XhoI and KpnI and blunt-ended with the use of a blunting kit (manufactured by Takara Shuzo Co., Ltd.). A pYEUra3 was cleaved with EcoRI and BamHI and blunt-ended with the use of a blunting kit. Then it was ligated to the blunt-ended PCR product (about 2.2 kb) described above. The plasmid thus obtained was named pYCscaur1R.

12-b) Construction of pYE vector carrying DNA coding for Aur1^{R}p (F158Y, A240C)

By using pUscaur1^R-C as a template, the DNA fragment (about 2.2 kb), which contained the DNA coding for Aur1^{R}p (F158Y, A240C), was amplified by the PCR method with the use of a primer represented by SEQ ID No. 49 in the Sequence Listing and a primer M13M4. The PCR

product was cleaved with BamHI and electrophoresed on an agarose gel. Then the target DNA fragment (about 1.8 kb) was recovered from the gel and purified. The plasmid pSCAR1 was cleaved with BamHI and thus a DNA fragment of 11.7 kb, from which a DNA fragment of 2.8 kb containing scaur1^S had been deleted, was obtained. This DNA fragment of 11.7 kb was ligated to the above-mentioned DNA fragment of 1.8 kb containing the DNA coding for Aur1^{R}p (F158Y, A240C). Then a plasmid having the fragment of 1.8 kb inserted thereinto in the desired direction was selected. This plasmid containing the DNA coding for Aur1^{R}p (F158Y, A240C) was named pWscaur1^R-C and employed in the transformation of a yeast for laboratory use.

Example 13: Transformation with the use of yeast as host

13-a) Transformation of sake yeast by pYCscaur1^R-C

By using 5 μg of a pYC plasmid pYCscaur1^R-C, Sake yeast Kyokai K-701 was transformed by the lithium acetate method described in Example 8-a). As Table 5 shows, the obtained results are similar to those obtained in the cases of the linearized plasmids. These transformants sustained each an aureobasidin A resistance at least 10 times higher than that of the parent strain even after being subcultured over several generations. Thus it has been confirmed that Aur1^{R}p (F158Y, A240C) is usable as an effective selective marker for sake yeast in the transformation by a replication vector.

Table 5

Plasmid	Pre-incubation time	No. of tranformants/μg DNA Aureobasidin A concn. (μg/ml) 0.5	1.0	2.0	5.0
pYCscaur1R	1 hour	0	0	0	0
	overnight	24	21	0	0
pYCscaur1^R-C	1 hour	386	172	18	1
	overnight	4824	4792	1692	136

13-b) Transformation of laboratory yeast by pWscaur1^R-C

By using a monoploid yeast DKD-5D for laboratory use (a, his3, trp1, leu2-3, 112) as a host, 5 μg of pWscaur1^R-C was transformed by the lithium acetate method described in Example 10-a).

For comparison, a transformant was screened on a minimal medium by using an auxotrophic marker contained in the plasmid. As Table 6 shows, it has been confirmed that Aur1^{R}p (F158Y, A240C) is usable as a selective marker which is comparable to the conventional auxotrophic markers in a monoploid yeast.

Table 6

Plasmid	Pre-incubation time	No. of transformants/μg DNA Minmal Aureobasidin A concn. (μg/ml) medium	0.5	1.0	5.0
pWscaur1^R-C	1 hour	192	248	181	58
	overnight	2500	2780	2524	2044

Example 14: Transformation by using *C. albicans* as host

14-a) 5 μg of a linear plasmid, which had been prepared by cleaving pUscaur1^R-C with SalI capable of cleaving at one point in the pUC119 region, was transformed into *C. albicans* TIMM0136 by the lithium acetate method. After the completion of the transformation, the cells were incubated in a YPD medium for 1 hour or overnight and then smeared on a YPD agar medium containing aureobasidin A. As Table 7 shows, transformants having the resistance to aureobasidin were obtained by effecting the pre-incubation overnight. These transformants showed each an MIC of 10 μg/ml or above.

Table 7

Plasmid	Pre-incubation time	No. or transformants/μg DNA Aureobasidin A concn. (μg/ml) 0.25	1.0
SalI-linearized	1 hour	0	0
pUscaur1^R-C	overnight	180	0

Example 15: Cloning of gene anaurl regulating aureobasidin sensitivity and originating in *A. nidulans*

15-a) Isolation of aureobasidin resistant mutant of *A. nidulans*

A strain *A. nidulans* FGSC89 showing a sensitivity to aureobasidin at 5 μg/ml was inoculated into an SD slant (containing 1% of polypeptone S, 2% of glucose and 2% of agar) and incubated therein at 30°C for 7 days. After suspending in 5 ml of a 0.1% Tween 80 solution containing 0.8% of NaCl, the suspension was filtered through a glass filter (3G3 type) and the obtained filtrate was used as a conidium suspension. This conidium suspension was UV-radiated for 5 minutes, thus effecting mutagenesis. Under these conditions, the rate of survival was about 25%. After blocking off the light for 30 minutes or longer, the conidia were inoculated into an SD plate and incubated at 30°C for 4 days. The conidia thus formed were collected with a glass filter, further inoculated into Cz+bi medium [containing 4.9% of Czapek solution agar (manufactured by Difco), 200 μg/ml of arginine and 0.02 μg/ml of biotin] containing 5 μg/ml of aureobasidin and incubated therein at 30°C. After 2 or 3 days, eight aureobasidin resistant colonies were obtained. Although these cells showed a resistance to 80 μg/ml of aureobasidin, they were the same as the parent strain in the sensitivities to amphotericin B, cycloheximide and clotrimazole. Thus it was estimated that the resistance thus acquired was not a multiple drug resistance but one specific to aureobasidin.

15-b) Preparation of genomic library of aureobasidin resistant strain

From a strain R1 showing a particularly high resistance from among the aureobasidin resistant strains, genomic DNAs were extracted and purified in the following manner. After incubating in a PD medium [containing 2.4% of potato dextrose broth (manufactured by Difco)] under shaking at 30°C for 2 days, the hyphae were collected with a glass filter (3Gl type) and

washed with distilled water. Then the cells were dehydrated and suspended in 20 ml of a protoplast generation solution [containing 20 μg/ml of Yatalase (manufactured by Ozeki Shuzo), 0.8 M of NaCl and 10 mM of a sodium phosphate buffer, pH 6.0]. Then the suspension was slowly stirred at 30°C overnight to thereby generate protoplasts. The suspension was filtered through a glass filter (3G2 type) and thus the protoplasts were collected into the filtrate and then harvested by centrifuging at 2,000 rpm for 5 minutes. After washing with 0.8 M NaCl twice, the protoplasts were suspended in 2 ml of a TE solution (containing 10 mM of Tris-HCl and 1 mM of EDTA, pH 8.0) and 2 ml of a lysis solution (containing 2% of SDS, 0.1 M of NaCl, 10 mM of EDTA and 50 mM of Tris-HCl, pH 7.0) was added thereto. After slowly stirring, the mixture was maintained at room temperature for 15 minutes and then centrifuged at 3,500 rpm for 10 minutes followed by the recovery of the supernatant. Then an equivalent amount of a mixture of phenol/chloroform/isoamyl alcohol (25/24/1) was added thereto and the mixture in the tube was gently mixed and centrifuged at 3,000 rpm for 5 minutes followed by the recovery of the upper liquid layer. Next, 2.5 times by volume as much ethanol at -20°C was added thereto. The resulting mixture was allowed to stand at -80°C for 10 minutes and then centrifuged at 3,500 rpm for 15 minutes. The DNAs thus precipitated were dried. Then 0.5 ml of a TE solution and 2.5 μl of an RNase A solution (20 μg/ml) were added thereto and the mixture was maintained at 37°C for 30 minutes.

After adding 0.5 ml of phenol/chloroform/isoamyl alcohol, the obtained mixture was gently mixed and centrifuged at 10,000 rpm for 5 minutes followed by the recovery of the upper layer. This procedure was repeated once. After adding 0.5 ml of chloroform/isoamyl alcohol (24/1), the obtained mixture was gently mixed and centrifuged at 10,000 rpm for 5 minutes followed by the recovery of the supernatant. Then 0.05 ml of 5 M NaCl and 0.5 ml of isopropyl alcohol were added and the mixture was allowed to stand at -80°C for 10 minutes and centrifuged at 10,000 rpm for 15 minutes to thereby collect DNAs.

Eight μg of the genomic DNAs thus purified were partially digested by treating with 4 U of a restriction enzyme BamHI at 37°C for 15 minutes. After deprotenization with phenol/chloroform, the DNA was recovered by ethanol precipitation. The DNAs were electrophoresed on a 0.8% agarose gel and DNAs in a region of from 3 to 15 kb were extracted and purified. The DNAs thus obtained were ligated to a vector pDHG25 [Gene, 98, 61-67 (1991)],

which had been completely digested with BamHI, with the use of a DNA ligation kit (manufactured by Takara Shuzo Co., Ltd.). Then *Escherichia coli,* HB101 was transformed thereby so as to prepare a genomic library of the resistant strain. The *E. coli* cells containing this genomic library were cultured in 50 ml of an LB medium (containing 1% of bactotrypton, 0.5% of bacto yeast extract and 0.5% of sodium chloride) containing 100 μg/ml of ampicillin at 37°C overnight. Next, plasmids were recovered and purified from the *E. coli* cells.

15-c) Expression and cloning of aureobasidin resistant gene anaurl^R

The plasmid originating in the genomic library of the aureobasidin resistant strain thus obtained was transformed into a strain *A. nidulans* FGSC89 by the following method. Namely, *A. nidulans* was incubated in a PD medium under shaking at 30°C for 2 days. Then the hyphae were collected by filtering the culture broth through a glass filter (3Gl type) and washed with sterilized water. After sufficiently dehydrating, the cells were suspended in 10 ml of a protoplast generation solution. After reacting by slowly shaking at 30°C for about 3 hours, the cell suspension was filtered through a glass filter 3G3. Then the filtrate was centrifuged at 2,000 rpm for 5 minutes to thereby collect the protoplasts therein. The collected protoplasts were washed with 0.8 M NaCl twice and suspended in Sol 1 (containing 0.8 M of NaCl, 10 mM of $CaCl_2$ and 10 mM of Tris-HCl, pH 8.0) in such a manner as to give a protoplast concentration of 2 x 10^8/ml. Then 0.2 time by volume as much Sol 2 [containing 40% (w/v) of PEG4000, 50 mM of $CaCl_2$ and 50 mM of Tris-HCl, pH 8.0] was added thereto and well mixed.

10 μg of the plasmid originating in the genomic library was added to a 0.2 ml portion of the protoplast suspension. After mixing well, the mixture was allowed to stand in ice for 30 minutes and then 1 ml of Sol 2 was added thereto. After mixing well, the mixture was allowed to stand at room temperature for 15 minutes and then 8.5 ml of Sol 1 was added thereto. After mixing well, the mixture was centrifuged at 2,000 rpm for 5 minutes to thereby collect the protoplasts. 0.2 ml of Sol 1 was added thereto and the resulting mixture was placed on the center of a minimum medium plate (containing 4.9% of Czapek solution agar, 0.8 M of NaCl and 0.02 μg/ml of biotin) containing 5 μg/ml of aureobasidin. Next, 5 ml of a soft agar medium (containing 3.5% of Czapek-Dox broth, 0.8 M of NaCl, 0.02 μg/ml of biotin and 0.5% of agar) was layered thereon followed by incubation at 30°C for 3 to 5 days. It was considered that the colonies growing on this plate

carried a plasmid containing an aureobasidin resistant gene.

Thus, about 70 colonies were formed on the aureobasidin-containing medium. These colonies were transplanted into 20 ml of a Cz+Bi medium and incubated at 30°C for 2 days. Then DNA was recovered and purified from the cells thus propagated in accordance with the method for the extraction and purification of DNA described in Example 15-b). A strain *E. coli* HB101 was transformed by this DNA and spread on an LB plate containing 100 μg/ml of ampicillin. Then a plasmid DNA was prepared from the *E. coli* colonies thus formed. This plasmid contained a DNA of 12 kb and was named pR1-1. Fig. 19 shows the restriction enzyme map of the 12 kb DNA contained in pR1-1. To further specify the resistant gene region, the DNA fragment of 12 kb was digested into fragments of various sizes with restriction enzymes. Next, these fragments were cloned into a vector pDHG25. Plasmids containing various DNAs were transformed into a strain *A. nidulans* FGSC89 so as to confirm whether the aureobasidin resistance could be thus acquired or not. As a result, it was revealed that the activity of imparting aureobasidin resistance resided in a fragment Bgl II (5.8 kb). Thus it was clarified that the gene anaurl^R was located in this fragment. Fig. 15 shows the restriction enzyme map of this DNA fragment containing the aureobasidin resistant gene anaurl^R. This fragment was subcloned into a vector pUC118 and the obtained plasmid was named pUR1. By using this plasmid, the DNA sequence of the DNA was identified. SEQ ID NO. 1 in the Sequence Listing shows this base sequence. As this DNA sequence indicates, the gene anaurl^R is one composed of two exon regions containing an intron. It has been revealed that this gene encodes a protein having the amino acid sequence represented by SEQ ID NO. 2 in the Sequence Listing.

15-d) Cloning of aureobasidin sensitive gene anaurl^s

To obtain the cDNA of the aureobasidin sensitive gene from normal cells of *A. nidulans*, total RNAs were first extracted from a strain *A. nidulans* FGSC89. Namely, this strain was incubated in 200 ml of a PD medium and the cells were collected with the use of a glass filter (3Gl type). After sufficiently dehydrating, the cells were quickly frozen with liquid nitrogen. Then the frozen cells were powdered in a mortar and total RNAs (2.6 mg) were extracted and purified with the use of an RNA extraction kit (manufactured by Pharmacia). From 1 mg of these RNAs, 12.8 μg of poly(A)$^+$RNAs were prepared by using Oligotex-dT30 <Super> (manufactured by Takara

Shuzo Co., Ltd.). By using 5 μg of the poly(A)$^+$RNAs, cDNAs were synthesized with the use of a Takara cDNA synthesizing kit (manufactured by Takara Shuzo Co., Ltd.). The cDNAs thus synthesized were ligated to a λ phage vector λSHlox™ (manufactured by Novagen, Inc.) and subjected to *in vitro* packaging with the use of Phage Maker™ System, Phage Pack Extract (manufactured by Novagen, Inc.) to thereby construct a cDNA library. This cDNA library was infected in a host strain *E. Coli* ER1647. After mixing with top agarose (an LB medium containing 0.7% of agarose), it was layered on an LB plate and incubated at 37°C overnight to thereby form plaques. The plaques thus formed were transferred onto a nylon membrane (Hybond-N, manufactured by Amersham) and subjected to plaque hybridization. As a probe, use was made of a DNA fragment of 2.6 kb obtained by cleaving the plasmid pUR1 obtained in Example 15-c) with PstI and SalI. This DNA fragment was labeled with [α-^{32}p]dCTP by using a random primer DNA labeling kit (manufactured by Takara Shuzo Co., Ltd.) and employed as a probe in the hybridization. As the result of screening of 4 x 10^5 plaques, 8 phage clones hybridizable with the probe were obtained. Next, these phages were subjected to automatic subcloning in *E. coli* to thereby give *E. coli* strains having plasmids wherein a cDNA-containing region had been automatically subcloned. The plasmids were purified from these strains and the cDNAs were compared in length. Thus pSl5 having the longest cDNA (2.9 kb) was selected and subcloned into pUC118 followed by the identification of the DNA sequence. This plasmid was named pANAR1. An *E. coli* strain transformed by pANAR1 was named *Escherichia coli* JM109/pANAR1 and has been deposited at National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology under the accession number FERM BP-5180. Fig. 16 shows the restriction enzyme map of this cDNA. The DNA base sequence thereof is represented by SEQ ID NO. 3 in the Sequence Listing. As this base sequence indicates, the gene anaurl^S encodes a protein having the amino acid sequence represented by SEQ ID NO. 4 in the Sequence Listing. A comparison with the resistant gene anaurl^R has revealed that the base G at the position 1218 in SEQ ID NO. 3 has been mutated into T and, at the amino acid level, the amino acid glycine at the position 275 has been converted into valine. It has been further clarified that the genomic DNA has one intron (56 bp). Fig. 19 shows the relation between the genomic DNA and cDNA.

Example 16: Confirmation and cloning of gene afaurl^S carried by *A. fumigatus*

16-a) Detection of gene afaurl^s by Northern hybridization

From a strain *A. fumigatus* TIMM1776, poly(A)$^+$RNAs were extracted and purified by the same method as the one of Example 15-d). The poly(A)$^+$RNAs (1 μg) of *A. fumigatus* and *A. nidulans* were separated by electrophoresing on a 1.2% agarose gel containing formaldehyde and transferred onto a nylon membrane. After fixing, hybridization was effected with the use of a HindIII fragment (741 bp) of the cDNA of the gene anaurl^s labeled with [α-^{32}p] dCTP as a probe. After hybridizing at 60°C overnight, the mixture was washed at 60°C with 0.5 x SSC and 0.1% SDS. In Fig. 19, the lanes 1 and 2 show the results of the hybridization of the poly(A)$^+$RNAs obtained from *A. nidulans* and *A. fumigatus* respectively. As Fig. 20 clearly shows, autoradiography of the hybridization revealed that *A. fumigatus* and *A. nidulans* both had the aureobasidin sensitive genes of the same size. However, the band of *A. fumigatus* was very weak, which indicates that the homology between these genes is not so high.

16-b) Cloning of gene afaurl^s carried by *A. fumigatus*

By using the poly(A)$^+$RNAs of *A. fumigatus* purified in Example 16-a), a cDNA library of *A. fumigatus* was prepared in accordance with the method for the preparation of a cDNA library described in Example 15-d). The above-mentioned library was screened under the same hybridization conditions as those of Example 16-a) with the use of a PstI-EcoRI fragment (921 bp) of the cDNA of *A. nidulans* as a probe. Thus eight phage clones were obtained. From these phase clone, the cDNA was recovered in the form of a plasmid by the method described in Example 15-d). The plasmids were purified and the cDNAs were compared in length. Thus the plasmid having the longest cDNA (2.9 kb) among them was selected. This cDNA was subcloned into pUC118 and the DNA sequence was identified. This base sequence, which is represented by SEQ ID NO. 12 in the Sequence Listing, indicates that this gene encodes a protein having the amino acid sequence represented by SEQ ID NO. 5 in the Sequence Listing. A comparison between the amino acid sequence of the afaurl^s protein of the strain *A. fumigatus* TIMM1776 with that of the anaurl^s protein of the strain *A. nidulans* FGSC 89 indicated that they had a high homology (87%).

Example 17: Confirmation of genes regulating aureobasidin sensitivity carried by *A. niger* and *A. oryzae*

Genomic DNAs were extracted and purified from strains *A. fumigatus* TIMM1776, *A. niger*

FGSC805 and *A. oryzae* IF05710 in accordance with the method described in Example 15-b). Further, genomic DNAs were extracted and purified from yeast strains *S. cerevisiae* DKD-5D and *Schizo. pombe* JY745 in accordance with the method of P. Philippsen et al. [Methods in Enzymology, 194, 169-175 (1991)]. 5 μg portions of the genomic DNAs of *A. nidulans*, *A. fumigatus*, *A. niger*, *A. oryzae*, *S. cerevisiae* and *Schizo. pombe* were cleaved with a restriction enzyme PstI, separated by electrophoresing on a 0.8% agarose gel, transferred onto a nylon membrane and fixed. Next, Southern hybridization was efected by using a PstI-EcoRI fragment of the cDNA of the anaurl^s gene labeled with [α-^{32}p]dCTP as a probe. The hybridization was effected under the same conditions as those described in Example 16-a). Fig. 21 shows the autoradiogram of the hybridization. As Fig. 21 clearly shows, genes regulating aureobasidin sensitivity occur in *A. niger* and *A. oryzae* too. It has been also revealed that the DNA of *A. nidulans* is not hybridizable with the aureobasidin sensitive genes of the yeasts *S. cerevisiae* and *Schizo. pombe*. In Fig. 21, the lanes 1, 2, 3, 4, 5 and 6 show the results of the Southern hybridization of the genomic DNAs of *A. nidulans*, *A. fumigatus*, *A. niger*, *A. oryzae*, *S. cerevisiae* and *Schizo. pombe* respectively.

According to the present invention, a novel protein regulating aureobasidin sensitivity and a gene coding for the protein, i.e., a gene regulating aureobasidin sensitivity are provided. These substances are useful in the diagnosis and treatment for diseases caused by organisms having the above-mentioned gene, such as mycoses. The present invention further provides an antisense DNA and an antisense RNA of this gene, a nucleic acid probe being hybridizable with the gene, a process for detecting the gene by using this nucleic acid probe, a process for producing a protein regulating aureobasidin sensitivity by using a transformant having the gene introduced thereinto, an antibody for the protein and a process for detecting the protein by using this antibody. They are also useful in the diagnosis and treatment of diseases including mycoses.

In addition, the present invention provides a chromosome integration vector with the use of a resistance to aureobasidin as a selective marker, which is useful in genetic recombination of fungi (in particular, industrial fungi), a process for producing a transformant having this vector introduced thereinto, and a transformant obtained by this process. They are effectively applicable to, for example the creation of a transformant for producing a useful protein and breeding. The

present invention also provides a protein capable of imparting a strong resistance to aureobasidin and a DNA coding for this protein. They imparted a resistance to an organism having an aureobasidin sensitivity and are useful as a selective marker for screening the organism thus acquiring the resistance. They are highly useful particularly in, for example, the genetic engineering breeding of a practically usable yeast and the analysis of genetic information of *C. albicans*. In particular, an aureobasidin resistant gene originating in *S. cerevisiae* is highly useful in the breeding of *S. cerevisiae* and the preparation of a transformant for producing a useful protein, because *S. cerevisiae* is a yeast which is not only widely employed as an industrial yeast but also highly safe in genetic recombination.

Both the aureobasidin-resistant genes obtained from yeasts and that from mold are isolated from the resistant mutant derived from yeasts and mold, respectively. And the aureobasidin-resistant gene of mold have function, giving an aureobasidin-resistance, identical to the genes of yeasts. That is, these genes are functional homologs. Although amino acid sequence of aureobasidin-resistant genes of yeasts have low homology with those of molds (for example, 35% in the comparison of that of spaurl and that of anaurl), middle regions of these amino acid sequences have high homology (57% in the comparison of the region between 24th and 329th of spaurl and the region between 59th and 364th of anaurl). Furthermore, a secondary structure predicted from amino acid sequence of these genes have identical characteristic which is membrane protein containing transmembrane domains. Therefore, these proteins are homologous in structure also.